Babesia vaccines

The invention relates to Babesia proteins of a 28kDa protein family and to immunogenic fragments thereof, to nucleic acids encoding such proteins, to cDNA fragments, recombinant DNA molecules, live recombinant carriers, and host cells, to vaccines, to methods for the preparation of such vaccines, to the use of such proteins or fragments, and to diagnostic tests.

Babesiosis, like malaria, is a disease, which has a geographically focal occurrence. The reason for this is that the pathogen is transmitted by ticks that feed on a certain reservoir of parasites present in a vertebrate population. Only where ticks are present, Babesiosis can occur. On balance, particularly in indigenous animals, the parasite coexists with the host without causing significant disease. In many cases Babesiosis becomes a problem because of man's activities through inbreeding of genetic traits and/or transporting animals to unfamiliar environments where Babesiosis is endemic (Callow, L.L. and Dalgliesh, R.J., 1982, in: "Immunology of Parasitic Infections", Cohen, S. and Warren, K.S. eds., p. 475-526, Blackwell Scientific).

Babesiosis also holds a threat as zoonotic agent, not only to immunocompromised humans (Gray *et al.*, 2002, Int. J. Med. Microbiol., vol. 291, p. 108-111).

Signs of disease in naturally acquired Babesiosis usually begin 7-21 days after infection. These symptoms include: fever, anorexia, depression, anaemia, haemoglobinuria and rapidly developing weakness. Increased lacrimation, salivation and muscle tremor commonly occur. Nervous signs may develop in terminal infections, and death may occur when the disease is left untreated. Coagulation disturbances lead to increased erythrocyte-stickiness. Thrombosis is not common, but small hyaline thrombi, connected with megakaryocytes have been described. As a result the blood passage through the microvasculature is hampered, resulting in congestion of internal organs and decreased packed cell volumes (PCV). This might impair the oxygen supply to certain tissues and subsequently lead to tissue damage as a result of anoxia.

Species from the Babesiidae have now been detected to infect most mammalian species of veterinary importance (Kuttler, K.L., in M. Ristic ed.: "Babesiosis of domestic animals and man". CRC Press, Inc., Boca Raton, FL, 1988): Cow (*B. divergens, B. bovis, B. bigemina*), Swine (*B. trautmanni, B. perroncitoi*), Sheep (*B. ovis, B. motasi*), Horse (*B. equi, B. caballi*), Dog (*B. canis, B. rossi, B. vogeli*), and Cat (*B. felis, B. cati*). In all these

species death or more or less severe economical losses (reduction in quality or quantity of meat, milk, wool, or offspring), or severe reduction in well being are caused either as a result of the Babesia infection directly, or through facilitation of secondary infections.

Medications exist to cure an established Babesia infection, for instance dogs can be treated with imidocarb dipropionate (commercially available as Carbesia ®) (Brandao *et al.*, 2003, Vet. Paras. vol. 114, p. 253-265). However such an injection is painful due to tissue irritation. Further it suffers the common drawbacks of such anti-parasitics: the prevention of a build up of immunological memory, potential toxicity, and build up of resistance.

It has been shown that Babesiosis can be controlled by vaccination with live vaccines (Pipano, 1995, Vet. Paras., vol. 57, p. 213-231). Such vaccines are produced by harvesting erythrocytes from infected animals. For some but not all Babesia species *in vitro* erythrocyte cultures have been developed, to increase the number of parasites. The infected erythrocytes from the animal or the cultures are then used to vaccinate animals.

General disadvantages of such live parasitic vaccines are that the inoculation material is largely uncontrolled, highly variable in its composition, biologically unsafe, and on the whole the process is unethical through the use of a large number of experimental animals. Additionally, Babesia parasites are very unstable; as they are strictly anaerobic, they must be kept away from oxygen or will die quickly.

Alternatively, not the parasite-infected erythrocytes themselves are used for vaccination, but the surrounding serum, or culture supernatant. Such surrounding liquids of infected erythrocytes contain so-called Soluble Parasite Antigens (SPA). Little is known about the composition of these preparations. It has been suggested that the protective activity is due to the immunising capacity of antigens of the merozoite surface coat in the serum or medium, a structure that is left behind during the process of invasion of the erythrocyte (Ristic, M. and Montenegro-James, S., 1988, In: "Babesiosis of Domestic Animals and Man", Ristic, M. ed., p. 163-190, CRC Press). In addition, during *in vitro* culture a number of parasites die, thereby (internal) parasitic antigens are released into the culture medium.

Such SPA preparations are capable of inducing an immune response that, although not necessarily affecting the parasite, sufficiently reduces the clinical manifestations of infection (Schetters and Montenegro-James, S., 1995, Parasitology today, vol. 11, p. 456-462). For instance SPA from culture supernatant of an *in vitro*

culture of Babesia canis parasite-infected erythrocytes induces protective immunity against homologous challenge infection.

An SPA vaccine for *Babesia canis* is available commercially as Pirodog®, and is prepared from the supernatant of a culture of a strain of *Babesia canis* (described in US patent 4,777,036). However, such a vaccine gives in general little protection against infections with (wild type) *B. canis* (Lepetit, C., 1988, "Piroplasmose canine et vaccination Pirodog", Doctoral Thesis, Univ. of Nantes, France).

In general, SPA based vaccines bear the same disadvantages as the live parasitic vaccines do, in that they are largely uncharacterised, highly variable and require many precautions to be biologically safe. Additionally the production of such vaccines is very difficult to scale up, as that requires the infection, housing and harvesting of experimental animals to provide parasites, erythrocytes, and/or serum.

It is an object of the invention to provide proteins or fragments thereof that can serve as effective subunit vaccines for infection with Babesiidae, that are well defined, safe, stable, with easily scaleable production.

It was surprisingly found now that a subunit vaccine comprising a member of a novel Babesia protein family; the 28 kDa protein family, as well as immunogenic fragments thereof incorporate all these advantageous characteristics.

Many disadvantages of live parasite- and SPA vaccines can now be overcome by the use of a member of this novel protein family or of immunogenic fragments thereof in protein subunit vaccines produced in an expression system; such a protein is highly defined, biologically safe, the product can be stabilized much better than whole live parasites, and its production can be easily scaled up

Proteins of the novel 28 kDa protein family are characterised in that they all share a specific amino acid sequence that is very well conserved amongst the various members of said family.

In spite of the presence of this well-conserved amino acid sequence, the overall length of the proteins of the 28 kDa protein family may well be different in the various members of the family of Babesiidae. Examples of members of the 28 kDa protein family are found to have a length ranging from below 26 kDa up to over 40 kDa.

Members of the 28 kDa protein family however all comprise a stretch of amino acids that has a level of homology of at least 70 % to the amino acid sequence from amino acid position 17 to position 180 in SEQ ID NO 2.

Merely as examples, in French *Babesia canis* isolate A, members of the 28 kDa family according to the invention, and further referred to as Bc28.1 or Bc28.2, were found to have the amino acid sequence as depicted in SEQ ID NO: 2 or 4 respectively.

The proteins of the 28kDa protein family are expressed from their respective encoding sequences that are members of a 28 kDa multigene family from Babesiidae, and have a high level of sequence identity at the nucleic acid level.

The proteins of the 28kDa protein family can be detected in infected erythrocytes by specific antisera. These sera recognize these specific proteins of the parasite also in Western blotting and immunoprecipitation experiments. Both proteins can be expressed in an expression system. Proteins or their fragments, expressed in this way can be used to formulate a subunit vaccine, which protects mammalians from (signs of) disease upon infection with species of Babesiidae.

The Bc28.1 protein exists in two forms; a free 26 kDa SPA form, which is present in Babesia infected erythrocytes and in their surrounding liquid; as well as a 28 kDa bound protein form that has a GPI anchor, and is associated with the membrane of the Babesia merozoite and with the outer membrane of the infected erythrocyte.

Because Babesiidae parasites spend most of their live hidden inside the erythrocytes, therefore an immune-response is most effective when focussed on antigens that can be 'seen' by the immune system. The 28 kDa form of Bc28.1 is such an antigen that is presented to the exterior, which allows an immune attack specifically directed to the infected erythrocyte. The 28 kDa form of the Bc28.1 protein binds to erythrocytes. This is indicative of a role in the agglutination of erythrocytes. As this process is a major cause of pathology and the way parasites infect new erythrocytes, interference at that level also provides effective immune intervention in disease progression.

Therefore, one aspect of the invention relates to a Babesia protein, characterised in that said protein comprises an amino acid sequence having a homology of at least 70% with the amino acid sequence from amino acid position 17 to position 180 in SEQ ID NO 2, or an immunogenic fragment of said protein.

In a preferred embodiment, the Babesia protein according to the invention is characterised in that said protein comprises an amino acid sequence having a homology of at least 70% with the amino acid sequence in SEQ ID NO 2, or an immunogenic fragment of said protein.

In another preferred embodiment, the Babesia protein according to the invention is characterised in that said protein comprises an amino acid sequence having a homology of at least 70% with the amino acid sequence in SEQ ID NO 4, or an immunogenic fragment of said protein.

In a more preferred embodiment, the Babesia protein according to the invention is characterised in that it is Bc28.1 protein, preferably in the 26 kDa or in the 28 kDa form.

In an another more preferred embodiment, the Babesia protein according to the invention is characterised in that it is Bc28.2 protein.

The term "protein" is meant to incorporate a molecular chain of amino acids. A protein is not of a specific length and can, if required, be modified *in vivo* or *in vitro*, by, e.g. glycosylation, amidation, carboxylation or phosphorylation. *Inter alia*, peptides, oligopeptides and polypeptides are included within the definition. A protein or peptide can be of biologic and/or synthetic origin.

A "Babesia protein" according to the invention is a protein which has a counterpart in an organism of the family Babesiidae.

Preferably the organism of the family Babesiidae is an organism selected from the group consisting of the species *Babesia divergens, B. bovis, B. motasi, B. caballi, B. equi, B. canis, B. rossi, B. vogeli, B. felis, B. cati, B. ovis, B. trautmanni, B. bigemina, B. microti,* and *B. gibsoni.*

More preferably the organism of the family Babesiidae is selected from the group consisting of the species *Babesia canis, B. rossi, B. caballi, B. equi, B. bovis,* and *B. bigemina.*

An "immunogenic fragment" is understood to be a fragment of a protein of the 28 kDa protein family that still has the capability to induce antibodies directed against such 28 kDa Babesia proteins.

Preferably an immunogenic fragment of a protein of the 28 kDa protein family according to the invention comprises at least 8 amino acids taken from the amino acid sequence of SEQ ID NO 2 or 4. More preferably such a fragment comprises 11, 15, 20, 30, 40, 50, 100, 150, or 200 amino acids taken from the amino acid sequence of SEQ ID NO 2 or 4, in that order of preference.

Preferably, an immunogenic fragment of a protein of the 28 kDa protein family according to the invention contains an epitope of such a protein. For instance an immunogenic fragment of a protein of the 28 kDa protein family according to the invention

is formed by a part of the protein that lacks the N-terminal signal sequence and/or the C-terminal GPI anchor sequence. Other fragments are for instance those comprising a specific epitope from a protein of the 28 kDa protein family. Such epitopes may be determined by the methods outlined below. All these immunogenic fragments are embodied in the invention.

An epitope is understood to be that part of an antigenic molecule to which a T-cell receptor will respond, or to which B-cells will produce antibodies. An epitope according to the invention will therefore induce specific T-cells or activate B-cells to produce specific antibodies such that these cells or antibodies give rise to an immune reaction that interferes with the course of an infection or disease. Thus, through such epitopes, an immune response can be generated.

In order to be antigenic, an amino acid fragments need to be of a certain length. Therefore an epitope consists of at least 8 – 11 amino acids for MHC I receptor binding, or of at least 11 – 15 amino acids for MHC II receptor binding (reviewed e.g. by R.N. Germain & D.H. Margulies, 1993, Annu. Rev. Immunol., vol. 11, p. 403-450, in: "The biochemistry and cell biology of antigen processing and presentation"). Amino acid fragments shorter than this may not be antigenic as such: they need to be coupled to a carrier, such as KLH, BSA or the like, using techniques known in the art. When coupled such short fragments may well be able to induce an immune response that is within the object of the invention.

Identification of immunogenic fragments or epitopes of a protein of the 28 kDa protein family according to the invention, can be easily performed by a variety of straightforward techniques, for instance by the so-called PEPSCAN method, or via computer algorithms that make comparisons to known epitopes.

The PEPSCAN method (WO 84/03564, and WO 86/06487, and H. Geysen *et al.*, Proc. Natl. Acad. Sci. USA, 1984, vol. 81, p. 3998-4002, and J. of Immunol. meth., 1987, vol. 102, p. 259-274), is an easy to perform, quick and well-established method for the detection of immunologic determinants of a protein. It comprises the synthesis of a series of peptide fragments progressively overlapping the protein under study, and subsequent testing of these polypeptides with specific antibodies to the protein. Such antibodies to the proteins according to the invention can be obtained by making polyclonal or monoclonal antibodies, by using techniques well known in the art.

The use of computer algorithms in the designation of specific protein fragments as the immunologically important epitopes on the basis of their sequential and/or structural agreement with epitopes that are known, is also a well known technique. The

determination of these regions can be based on a combination of the hydrophilicity criteria according to Hopp and Woods (1981, Proc. Natl. Acad. Sci. USA, vol. 78, p. 3824-3828), and the secondary structure aspects according to Chou and Fasman (1987, Advances in Enzymology, vol. 47, p. 45-148, and US patent 4,554,101). Immunogenic epitopes can likewise be predicted from the protein's amino acid sequence by computer with the aid of Berzofsky's amphiphilicity criterion (1987, Science, vol. 235, p. 1059-1062 and US patent application NTIS US 07/005,885). A condensed overview of the use of these methods is found in Shan Lu (common principles; 1991, Tibtech, vol. 9, p. 238-242), Good *et al.* (Malaria epitopes; 1987, Science, vol. 235, p. 1059-1062), Lu (review; 1992, Vaccine, vol. 10, p. 3-7), and Berzofsky (HIV-epitopes; 1991, The FASEB Journal, vol. 5, p. 2412-2418).

An illustration of the effectiveness of using these methods was published by H. Margalit *et al.* (1987, J. of Immunol., vol. 138, p. 2213-2229) who describe success rates of 75 % in the prediction of T-cell epitopes using such methods.

The percentage of homology between the proteins according to the invention is determined with the computer program "BLAST 2 SEQUENCES" by selecting sub-program: "BlastP" (T. Tatusova & T. Madden, 1999, FEMS Microbiol. Letters, vol. 174, p. 247-250), that can be found at www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html. The comparison-matrix that is used is: "blosum62", with the default parameters: open gap penalty: 11; extension gap penalty: 1, and gap x_dropoff: 50.

This program lists the percentage of amino acids that are identical as "Identities", and the percentage of amino acids that are homologous as "Positives"

For example, the amino acid sequences of Bc28.1 and Bc28.2 are aligned in Figure 1. A high percentage of homology exists between the two proteins, especially in the N-terminal 3/4^{rs}. The percentage homologies (the percentage of "positives" from the BlastP program) are presented in Table 1.

	Amino acids	Percentage homology
Complete	244	91
N-terminal	180	97
C-terminal	64	73

Table 1: Results of BlastP amino acid alignment of Bc28.1 and Bc28.2 proteins.

It will be understood that, for a particular protein of the 28 kDa protein family, natural variations exist between the proteins associated with individual strains or species of Babesiidae. These variations may be demonstrated by (an) amino acid difference(s) in the overall sequence or by deletions, substitutions, insertions, inversions or additions of (an) amino acid(s) in said sequence. Amino acid substitutions, which do not essentially alter biological and immunological activities, have been described, e.g. by Neurath *et al.*, 1979, in: "The Proteins", Academic Press New York. Amino acid replacements between related amino acids or replacements which have occurred frequently in evolution are, i.a. Ser/Ala, Ser/Gly, Asp/Gly, Asp/Asn, Ile/Val (see Dayhof, M.D., 1978, "Atlas of protein sequence and structure", Nat. Biomed. Res. Found., Washington D.C. vol. 5, suppl. 3). Other common amino acid substitutions include Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Thr/Phe, Ala/Pro, Lys/Arg, Leu/Ile, Leu/Val and Ala/Glu. Based on this information, Lipman and Pearson developed a method for rapid and sensitive protein comparison (1985, Science, vol. 227, p. 1435-1441) and determining the functional similarity between homologous proteins. Such amino acid substitutions of the exemplary embodiments of this invention, as well as variations having deletions and/or insertions are within the scope of the invention as long as the resulting proteins retain immune reactivity. Those variations in the amino acid sequence of a certain protein according to the invention that still provide a protein capable of inducing an immunological response against an organism of the family Babesiidae are considered as "not essentially influencing the immunogenicity", and are an embodiment of the invention.

This explains why proteins of the 28 kDa protein family according to the invention, when isolated from different species, may have homology percentages of 70 or more to the amino acid sequences in SEQ ID NO: 2 or 4, while still representing the same protein with the same immunological characteristics, i.e. the capability of inducing an immunological response against an organism of the family Babesiidae.

Proteins of the 28 kDa protein family according to the invention can be obtained from member species of the Babesiidae family.

However in an even more preferred embodiment, the proteins of the 28 kDa protein family according to the invention or immunogenic fragments thereof are characterised in that they are obtained from *B. divergens, B. bovis, B. motasi, B. caballi, B. equi, B. canis, B. rossi, B. vogeli, B. felis, B. cati, B. ovis, B. trautmanni, B. bigemina, B. microti,* or *B. gibsoni.*

Still even more preferably the proteins of the 28 kDa protein family according to the invention or immunogenic fragments thereof are characterised in that they are obtained from *Babesia canis, B. rossi, B. caballi, B. equi, B. bovis,* or *B. bigemina.*

With respect to the current taxonomic classification, the skilled person will realise this may change over time, as new insights lead to reclassification into new groups or to addition to existing groups. However, as this does not change the protein repertoire of the organism involved, only its classification, such re-classified organisms are considered to be embodied by the invention. For example *B. canis* and *B. rossi* were formerly classified as subspecies *B. canis canis* and *B. canis rossi.*

Sibinovic K., *et al.* (1967, J. of Paras., vol. 53, p. 919-923) studied isolated Babesia antigens from serum of horses infected with *Babesia equi* and *B. caballi,* and from dogs infected with *B. canis.* Significant similarities were noted in the biochemical characteristics of the antigens from these species. The Babesia proteins of the 28 kDa protein family according to the invention as obtained from *B. canis* will therefore also be present in *B. equi* and *B. caballi,* and in other species of the Babesiidae family.

In Example II, section 2.2.5. and Figure 11 the specific recognition of the 26 and 28 kDa forms of Bc28.1 protein from *B. canis* by an antiserum against *B. rossi* is disclosed. This illustrates proteins of the 28 kDa protein family present in *B. rossi* are immunologically related to those in *B. canis.*

Bc28.1 coding sequences have been obtained from geographically and genetically disparate *B. canis* field isolates. The deduced amino acids of a few examples of such isolates are aligned in Figure 2. The percentage homologies ("Positives") determined by pairwise alignment using the BlastP program are presented in Table 2.

	Robin	A8	B	34.01
A8	100			
B	98	98		
34.01	97	97	98	
Castres	98	98	97	99

Table 2: Percentage homologies of pair-wise BlastP amino acid alignments between the complete Bc28.1 proteins from geographically and genetically disparate *B. canis* field isolates. Bc28.1 proteins were 255 or 256 amino acids long.

Therefore, in the most preferred embodiment the invention relates to a Bc28.1 or a Bc28.2 protein or an immunogenic fragment of said proteins, characterised in that said proteins or immunogenic fragments are obtained from a *B. canis* isolate selected from the group consisting of A8, B, 34.01, A, Robin, and Castres.

A protein named Bd37, from *Babesia divergens* has been described before (EP 1050541 A1). At first instance Bd37 may appear to resemble the proteins Bc28.1 and Bc28.2. However, Bd37 and the two Bc28 proteins are totally unrelated:

- there is no significant sequence similarity between Bd37 protein or its coding sequence, and either of the two Bc28 proteins or their coding sequences.
- an antiserum against Bd37 does not recognize the 26/28 and 45 kDa proteins of *B. canis* that are recognized by antibodies against Bc28.1 and 28.2 respectively, neither in Western blot nor in immunoprecipitation experiments; for instance an antiserum against Bd37-His protein does not recognize a GST-Bc28.2 protein (Figure 10, B, lane 6).
- whereas both Bd37 and Bc28.1 are attached to the erythrocyte's outer membrane, Bd37 can be eluted off with 0.5 M NaCl, while Bc28.1 remains associated even at elution with 2 M NaCl (Example II, sections 2.1.5.1. and 2.2.6.1.)

The preferred way to produce the proteins of the 28 kDa protein family according to the invention is by using genetic engineering techniques and recombinant expression systems. These may comprise using nucleic acids, cDNA fragments, recombinant DNA molecules, live recombinant carriers, and/or host cells.

Therefore, another aspect of the invention relates to a nucleic acid, characterised in that it encodes the proteins of the 28 kDa protein family according to the invention, or an immunogenic fragment of said protein.

In a preferred embodiment the nucleic acid according to the invention comprises the nucleic acid of SEQ ID NO: 1.

In another preferred embodiment the nucleic acid according to the invention comprises the nucleic acid of SEQ ID NO: 3.

The term "nucleic acid" is meant to incorporate a molecular chain of desoxy- or ribonucleic acids. A nucleic acid is not of a specific length, therefore polynucleotides, genes, open reading frames (ORF's), probes, primers, linkers, spacers and adaptors are included within the definition. A nucleic acid can be of biologic and/or synthetic origin. The nucleic acid may be in single stranded or double stranded form. The single strand may be in sense or anti-sense orientation. Also included within the definition are modified RNAs or DNAs. Modifications in the bases of the nucleic acid may be made, and bases such as Inosine may be incorporated. Other modifications may involve, for example, modifications of the backbone.

The term "encodes" is meant to incorporate providing the possibility of protein expression, i.a. through transcription and/or translation when brought into the right context.

A nucleic acid according to the invention encodes a protein of the 28 kDa protein family according to the invention, or encodes an immunogenic fragment of said protein.

A nucleic acid according to the invention has a minimal length of 24 nucleotides taken from the nucleic acid sequence of SEQ ID NO 1 or 3, preferably a nucleic acid according to the invention comprises 50, 100, 250, or 500 nucleotides taken from the nucleic acid sequence of SEQ ID NO 1 or 3, in that order of preference.

A nucleic acid according to the invention for instance is a nucleic acid encoding a protein of the 28 kDa protein family according to the invention without a signal sequence and/or a GPI anchor. Other nucleic acids may comprise a sequence encoding a specific epitope of a protein of the 28 kDa protein family. Such nucleic acids are all embodied in the invention.

The percentage of identity between nucleic acids according to the invention is determined with the computer program "BLAST 2 SEQUENCES" by selecting sub-program: "BlastN" (T. Tatusova & T. Madden, 1999, FEMS Microbiol. Letters, vol. 174, p. 247-250), that can be found at www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html. Parameters that are used are the default parameters: reward for a match: +1; penalty for a mismatch: -2; open gap penalty: 5; extension gap penalty: 2; and gap x_dropoff: 50. Unlike the output of the BlastP program described above, the BlastN program does not list homologies, but identities; the percentage of nucleotides that are identical are indicated as "Identities".

It is well known in the art, that many different nucleic acids can encode one and the same protein. This is a result of what is known in molecular biology as "wobble", or the "degeneracy of the genetic code"; when several codons or triplets of mRNA will cause the same amino acid to be attached to the chain of amino acids growing in the ribosome during translation. It is most prevalent in the second and especially the third base of each triplet encoding an amino acid. This phenomenon can result in a heterology of about 30% for two different nucleic acids that still encode the same protein. Therefore, two nucleic acids having a nucleotide sequence identity of about 70 % can still encode one and the same protein.

For example, the nucleic acids encoding the Bc28.1 and Bc28.2 proteins according to the invention are aligned in Figure 3. A high percentage of identity exists between the two coding sequences, especially in the 5' 3/4^{rs} of the coding sequence. The percentage identities from the BlastN program are presented in Table 3.

	Nucleotides	Percentage identity
Complete	845	94
5'	652	97
3'	193	81

Table 3: Results of BlastN nucleotide sequence alignments between the nucleic acids encoding the Bc28.1 and Bc28.2 proteins according to the invention.

Nucleic acids encoding the proteins of the 28 kDa protein family according to the invention can be obtained from member species of the Babesiidae family.

However in a more preferred embodiment, the nucleic acids encoding a protein of the 28 kDa protein family or immunogenic fragments thereof according to the invention are characterised in that they are obtained from *B. divergens, B. bovis, B. motasi, B. caballi, B. equi, B. canis, B. rossi, B. vogeli, B. felis, B. cati, B. ovis, B. trautmanni, B. bigemina, B. microti,* or *B. gibsoni.*

In an even more preferred embodiment the nucleic acids encoding a protein according to the 28 kDa protein family or immunogenic fragments thereof are characterised in that they are obtained from *Babesia canis, B. rossi, B. caballi, B. equi, B. bovis,* or *B. bigemina.*

The possibility of species being taxonomically re-classified has been discussed above. As this does not change the organism's genome, such reclassified organisms are also embodied in the invention.

Also embodied in the invention are proteins of the 28 kDa protein family and nucleic acids encoding them from non-mammalian Babesiidae; such proteins or genes are present due to the high conservation of the proteins of the 28 kDa protein family, their encoding sequences, their genes, and gene-family.

Nucleic acids encoding Bc28.1 protein have been obtained from geographically and genetically disparate *B. canis* field isolates. The nucleotide sequences of a few examples of such isolates are aligned in Figure 4. The percentage identities of pairwise BlastN alignments are presented in Table 4.

	Robin	A8	B	34.01
A8	100			
B	98	98		
34.01	97	97	98	
Castres	98	98	98	98

Table 4: Percentage identities of pair-wise BlastN nucleotide sequence alignments between the nucleic acids encoding Bc28.1 proteins from geographically and genetically disparate *B. canis* field isolates. These nucleic acids were 849 or 852 nucleotides long.

Nucleic acids encoding the proteins of the 28 kDa protein family according to the invention can be obtained, manipulated and expressed by standard molecular biology techniques that are well-known to the skilled artisan, and are explained in great detail in standard text-books like Sambrook & Russell: "Molecular cloning: a laboratory manual" (2001, Cold Spring Harbour Laboratory Press; ISBN: 0879695773). One such type of manipulations is the synthesis of a cDNA fragment from RNA, preferably from mRNA which can be isolated from parasites, or parasite- infected cells or -organisms by techniques known in the art.

Therefore, in another preferred embodiment, the invention relates to a cDNA fragment according to the invention.

The preferred method of obtaining a cDNA fragment by reverse transcription is through a polymerase chain reaction (PCR) technique. Standard techniques and protocols for performing PCR are for instance extensively described in C. Dieffenbach & G. Dveksler: "PCR primers: a laboratory manual" (1995, CSHL Press, ISBN 879694473).

In still another preferred embodiment, the invention relates to a recombinant DNA molecule comprising a nucleic acid according to the invention, or a cDNA fragment according to the invention, under the control of a functionally linked promoter.

To construct a recombinant DNA molecule according to the invention, preferably DNA plasmids are employed. Such plasmids are useful e.g. for enhancing the amount of DNA-insert, as a probe, and as tool for further manipulations. Examples of such plasmids for cloning are plasmids of the pBR, pUC, and pGEM series; all these are available from several commercial suppliers.

The nucleic acid encoding a protein of the 28 kDa protein family according to the invention or an immunogenic fragment of said protein, can be cloned into separate plasmids and be modified to obtain the desired conformation using techniques well known in the art. However they may also be combined into one construct for improved cloning or expression purposes.

Modifications to the coding sequences encoding a protein of the 28 kDa protein family according to the invention may be performed e.g. by using restriction enzyme digestion, by site directed mutations, or by polymerase chain reaction (PCR) techniques.

For the purpose of protein purification or -detection, or improvement of expression level, additional nucleic acids may be added. This may result in the final nucleic acid comprised in the cDNA fragment, or in the recombinant DNA molecule being larger than the sequences required for encoding a protein of the 28 kDa protein family. When such additional elements are inserted in frame, these become an integral part of the protein of the 28 kDa protein family that is expressed. Such fused proteins are also embodied in the invention

An essential requirement for the expression of a nucleic acid, cDNA fragment, or recombinant DNA molecule is that these are operably linked to a transcriptional regulatory sequence such that this is capable of controlling the transcription of the nucleic acid, cDNA, or recombinant DNA. Transcriptional regulatory sequences are well known in the art and comprise i.a. promoters and enhancers. It is obvious to those skilled in the art that the choice of a promoter extends to any eukaryotic, prokaryotic or viral promoter capable

of directing gene transcription, provided that the promoter is functional in the expression system used.

In yet another preferred embodiment, the invention relates to a live recombinant carrier comprising a nucleic acid according to the invention, a cDNA fragment according to the invention, or a recombinant DNA molecule according to the invention.

Such live recombinant carriers (LRC's) are e.g. micro-organisms such as bacteria, parasites and viruses in which additional genetic information has been cloned, in this case a nucleic acid, a cDNA, or a recombinant DNA molecule, encoding a protein of the 28 kDa protein family according to the invention or an immunogenic fragment thereof. Target mammalians inoculated with such LRCs will produce an immunogenic response not only against the immunogens of the carrier, but also against the heterologous protein(s) or immunogenic fragment(s) for which the genetic code is additionally cloned into the LRC, e.g. a sequence encoding a protein of the 28 kDa protein family, or an immunogenic fragment thereof.

As an example of bacterial LRCs, attenuated *Salmonella* strains known in the art can attractively be used.

Alternatively, live recombinant carrier parasites have i.a. been described by Vermeulen, A. N. (1998, Int. Journ. Parasitol., vol. 28, p. 1121-1130).

LRC viruses may be used as a way of transporting a nucleic acid into a target cell. Live recombinant carrier viruses are also called vector viruses. Viruses often used as vectors are Vaccinia viruses (Panicali *et al.*, 1982, Proc. Natl. Acad. Sci. USA, vol. 79, p. 4927), Herpesviruses (EP 0473210-A2), and Retroviruses (Valerio, D. *et al.*, 1989, in: Baum, S.J., Dicke, K.A., Lotzova, E. and Pluznik, D.H. (Eds.), "Experimental Haematology today", Springer Verlag, New York: pp. 92-99).

The technique of *in vivo* homologous recombination, well known in the art, can be used to introduce a recombinant nucleic acid according to the invention into the genome of an LRC bacterium, parasite or virus of choice, capable of inducing expression of the inserted nucleic acid, cDNA or recombinant DNA according to the invention in the host animal.

Bacterial, yeast, fungal, insect, and vertebrate cell expression systems are used as host cells for expression purposes very frequently. Such expression systems are well known in the art and generally available, e.g. commercially through Invitrogen (the Netherlands).

Therefore, in yet still another preferred embodiment, the invention relates to a host cell comprising a nucleic acid according to the invention, a cDNA fragment according to the invention, a recombinant DNA molecule according to the invention, or a live recombinant carrier according to the invention.

A host cell to be used for expression of a protein of the 28 kDa protein family according to the invention may be a cell of bacterial origin, e.g. from *Escherichia coli*, *Bacillus subtilis*, *Lactobacillus sp.* or *Caulobacter crescentus*, in combination with the use of bacteria-derived plasmids or bacteriophages for expressing the sequence encoding the Bc28 protein. The host cell may also be of eukaryotic origin, e.g. yeast-cells in combination with yeast-specific vector molecules, or higher eukaryotic cells, like insect cells (Luckow et al,1988, Bio-technology, vol. 6, p. 47-55) in combination with vectors or recombinant baculoviruses; plant cells in combination with e.g. Ti-plasmid based vectors or plant viral vectors (Barton, K.A. *et al.*, 1983, Cell, vol. 32, p. 1033); or mammalian cells like Hela cells, Chinese Hamster Ovary cells or Crandell-Rees feline kidney-cells, also with appropriate vectors or recombinant viruses.

Next to these expression systems, plant cell, or parasite-based expression systems are attractive expression systems. Parasite expression systems are e.g. described in the French Patent Application, publication number 2 714 074, and in US NTIS publication no. US 08/043109 (Hoffman, S. & Rogers, W., 1993). Plant cell expression systems for polypeptides for biological application are e.g. discussed in R. Fischer *et al.* (1999, Eur. J. of Biochem., vol. 262, p. 810-816), and J. Larrick *et al.* (2001, Biomol. Engin., vol. 18, p. 87-94).

Expression may also be performed in so-called cell-free expression systems. Such systems comprise all essential factors for expression of an appropriate recombinant nucleic acid, operably linked to a promoter that will function in that particular system. Examples are the *E. coli* lysate system (Roche, Basel, Switzerland), or the rabbit reticulocyte lysate system (Promega corp., Madison, USA).

The protein of the 28 kDa protein family according to the invention or immunogenic fragments of said protein is very well suited for the production of a protein subunit vaccine. Such proteins or fragments can be obtained from parasites, or from animals or cells infected with Babesiidae parasites. However, much more convenient is the use of the nucleic acids encoding the protein of the 28 kDa protein family according to the invention or an immunogenic fragment of said protein, in an expression system. This is followed by harvesting the proteins or fragments produced and formulating these into a protein subunit

vaccine, e.g. by admixing a protein of the 28 kDa protein family according to the invention or an immunogenic fragment of said protein, and a pharmaceutically acceptable carrier.

Therefore, yet another aspect of the invention relates to a vaccine comprising a protein according to the invention or an immunogenic fragment of said protein, a nucleic acid, a cDNA fragment, a recombinant DNA molecule, a live recombinant carrier, or a host cell according to the invention, or a combination thereof, and a pharmaceutically acceptable carrier.

As described above, a protein of the 28 kDa protein family or an immunogenic fragment of said protein can advantageously be used for vaccination. If such proteins or fragments do not produce an immune response on their own, they can be coupled to a carrier such as KLH, BSA or the like.

The coupling of protein or fragments thereof can also be done to enhance or modify the immune response induced. For instance it is common practice to couple protein(-fragment)s to Tetanus toxoid to enhance the response of T-cells. Also specific effector molecules may be added, such as a toxin, to improve the killing of target cells. Such couplings can be performed

- chemically, by coupling, conjugation or cross-linking, through dehydration, esterification, etc, of the amino acid sequences either directly or through an intermediate structure.
- physically, by coupling through capture in or on a macromolecular structure, or preferably
- by molecular biological fusion, through the combination of recombinant nucleic acid molecules which comprise fragments of nucleic acid capable of encoding each of the two, such that a single continuous expression product is finally produced. Molecular engineering techniques are preferred.

An alternative and efficient way of vaccination is by direct vaccination with DNA encoding the relevant antigen or epitope. Direct vaccination with DNA encoding proteins has been successful for many different proteins, as reviewed in e.g. Donnelly *et al.* (1993, The Immunologist, vol. 2, p. 20-26). For example in the field of anti-parasite vaccines, protection against e.g. *Plasmodium yoelii* has been obtained with DNA-vaccination with the *P. yoelii* circumsporozoite gene (Hoffman, S. *et al.*, 1994, Vaccine, vol. 12, p. 1529-1533), and protection against *Leishmania major* has been obtained with DNA-vaccination

with the *L. major* surface glycoprotein gp63 gene (Xu & Liew, 1994, Vaccine, vol. 12, p. 1534-1536).

Such a DNA vaccination can be performed with a nucleic acid, a cDNA fragment, or preferably with a recombinant DNA molecule according to the invention.

Therefore, one preferred embodiment relates to a vaccine according to the invention, characterised in that it comprises a nucleic acid, a cDNA fragment, or a recombinant DNA molecule according to the invention.

Alternatively, a vaccine according to the invention can comprise live recombinant carriers as described above, capable of expressing the protein of the 28 kDa protein family according to the invention or immunogenic fragments of said protein. Such vaccines, e.g. based upon a bacterial, a parasitic or a viral carrier or vector have the advantage over subunit vaccines that they better mimic the natural way of infection by Babesiidae. Also the presentation of the antigens by cells infected with the carriers resembles the route proteins of the 28 kDa protein family or their immunogenic fragments are presented to the immune system in a natural infection. Moreover, their self-propagation is an advantage since only low amounts of the recombinant carrier are necessary for immunisation.

Thus, another preferred embodiment relates to a vaccine according to the invention, which comprises a live recombinant carrier and a pharmaceutically acceptable carrier.

The host cells as described above can be used to express a protein of the 28 kDa protein family according to the invention or an immunogenic fragment of said protein as an expression system. After expression the proteinacious product may be harvested, but alternatively the culture medium or the complete host cells themselves may be used in a vaccine. This has the benefit of omitting purification steps, but of course requires some tolerance by the target mammalians for the media components and/or components of the host cells.

Also embodied in the invention is a vaccine according to the invention devised of a combination from two or more types of molecules from the protein or immunogenic fragment thereof, nucleic acid, cDNA, recombinant molecule, live recombinant carrier, and host cells according to the invention. These may be combined in a single dose or in separate doses, and may be given at the same time or sequentially. For instance, a combination vaccination of an initial priming with a recombinant DNA plasmid carrying the

coding sequence of a protein of the 28 kDa protein family, followed some time later by a booster vaccination with a protein of the 28 kDa protein family may advantageously be used.

Vaccines according to the invention, can be administered in amounts containing between 0.1 and 1000 µg of a protein of the 28 kDa protein family according to the invention or an immunogenic fragment of said protein per mammalian target. Smaller or larger doses can in principle be used; preferably a dose of between 50 and 200 µg of a protein of the 28 kDa protein family or an immunogenic fragment thereof is used .

For live viral vector vaccines the dose rate per animal may range from 1 to 10^{10} pfu, preferably $10 - 10^5$ pfu are used.

A pharmaceutically acceptable carrier is understood to be a compound that does not adversely effect the health of the animal to be vaccinated, at least not to the extend that the adverse effect is worse than the effects seen when the animal is not vaccinated. A pharmaceutically acceptable carrier can be e.g. sterile water or a sterile physiological salt solution. In a more complex form the carrier can e.g. be a buffer.

Often, a vaccine is mixed with stabilizers, e.g. to protect degradation-prone components from being degraded, to enhance the shelf-life of the vaccine, or to improve freeze-drying efficiency. Useful stabilizers are i.a. SPGA (Bovarnik *et al.*, 1950, J. Bacteriology, vol. 59, p. 509), skimmed milk, gelatine, bovine serum albumin, carbohydrates e.g. sorbitol, mannitol, trehalose, starch, sucrose, dextran or glucose, proteins such as albumin or casein or degradation products thereof, and buffers, such as alkali metal phosphates.

The vaccine according to the invention may additionally comprise a so-called "vehicle". A vehicle is a compound to which the proteins, protein fragments, nucleic acids or parts thereof, cDNA's, recombinant molecules, live recombinant carriers, and/or host cells according to the invention adhere, without being covalently bound to it. Such vehicles are i.a. bio-microcapsules, micro-alginates, liposomes, macrosols, aluminium-hydroxide, -phosphate, -sulphate or -oxide, silica, Kaolin®, and Bentonite®, all known in the art.

An example is a vehicle in which the antigen is partially embedded in an immune-stimulating complex, the so-called ISCOM® (EP 109.942, EP 180.564, EP 242.380).

In addition, the vaccine according to the invention may comprise one or more suitable surface-active compounds or emulsifiers, e.g. Span® or Tween®.

For reasons of e.g. stability or economy the proteins, immunogenic fragments thereof, nucleic acids, cDNA's, recombinant molecules, live recombinant carriers, host cells and vaccines according to the invention may be freeze-dried. In general this will enable prolonged storage at temperatures above zero ° C, e.g. at 4°C.

Procedures for freeze-drying are known to persons skilled in the art; equipment for freeze-drying at different scales is available commercially.

Therefore, in a more preferred embodiment, the vaccine according to the invention is characterised in that it is in a freeze-dried form.

To reconstitute the freeze-dried vaccine, it may be suspended in a physiologically acceptable diluent. Such a diluent can e.g. be as simple as sterile water, or a physiological salt solution. In a more complex form it may be suspended in an emulsion as outlined in PCT/EP99/10178.

Target subjects for the vaccine according to the invention are preferably mammalian, e.g. humans or mammalian animals of veterinary importance. The target may be healthy or diseased, and may be seropositive or -negative for Babesiidae parasites or for antibodies to Babesiidae parasites. The target subject can be of any age at which it is susceptible to the vaccination and/or to the infection or clinical disease the vaccination aims to protect against.

The more preferred target mammalians for the vaccine according to the invention are cows, horses, dogs and cats.

The vaccine according to the invention can equally be used as prophylactic and as therapeutic treatment, and interferes with the establishment and/or with the progression of an infection or its clinical symptoms of disease.

The vaccine according to the invention can be in several forms, e.g.: a liquid, a gel, an ointment, a powder, a tablet, or a capsule, depending on the desired method of application to the target.

Preferably the vaccine is in the form of an injectable liquid.

The vaccine according to the invention can be administered to the mammalian target according to methods known in the art. For instance by parenteral applications such as through all routes of injection into or through the skin: e.g. intramuscular, intravenous, intraperitoneal, intradermal, submucosal, or subcutaneous. Alternative routes of

application that are feasible are by topical application as a drop, spray, gel or ointment to the mucosal epithelium of the eye, nose, mouth, anus, or vagina, or onto the epidermis of the outer skin at any part of the body; by spray as aerosol, or powder. Alternatively, application can be via the alimentary route, by combining with the food, feed or drinking water e.g. as a powder, a liquid, or tablet, or by administration directly into the mouth as a liquid, a gel, a tablet, or a capsule, or to the anus as a suppository.

The preferred application route is by intramuscular or by subcutaneous injection.

It goes without saying that the optimal route of application will depend on the particularities of the parasitic infection or clinical disease that is to be prevented or ameliorated, and the characteristics of the vaccine formulation that is used.

The scheme of the application of the vaccine according to the invention to the target mammalian can be in single or multiple doses, which may be given at the same time or sequentially, in a manner compatible with the dosage and formulation, and in such an amount as will be immunologically effective.

Preferably the vaccine is applied in one single dose that will provide sufficient immunological protection for at least a year.

In an even more preferred embodiment, the vaccine according to the invention is characterised in that it comprises an adjuvant.

An adjuvant in general is a substance that boosts the immune response of the target in a non-specific manner. Many different adjuvants are known in the art. Examples of adjuvants are Freund's Complete and -Incomplete adjuvant, vitamin E, non-ionic block polymers and polyamines such as dextransulphate, carbopol and pyran. Also very suitable are saponins, which are the preferred adjuvant. Saponins are preferably added to the vaccine at a level between 10 and 10.000 µg/ml. Within the group of saponins, the saponin Quil A® is the more preferred adjuvant. Saponin and vaccine components may be combined in ISCOMS® (EP 109.942, EP 180.564, EP 242.380).

Furthermore, peptides such as muramyldipeptides, dimethylglycine, or tuftsin, are often used as adjuvant, and mineral oil e.g. Bayol® or Markol®, vegetable oils or emulsions thereof and DiluvacForte® can advantageously be used.

It goes without saying that other ways of adjuvating, adding vehicle compounds or diluents, emulsifying or stabilizing a vaccine are also embodied in the invention. Such additions are for instance described in well-known handbooks such as: "Remington: the science and practice of pharmacy" (2000, Lippincot, USA, ISBN: 683306472), and:

"Veterinary vaccinology", P. Pastoret *et al.* ed., 1997, Elsevier, Amsterdam, ISBN 0444819681).

The vaccine according to the invention can advantageously be combined with another antigen, or immunoactive component. This can also be added in the form of its encoding nucleic acid.

Therefore, in a still even more preferred embodiment the vaccine according to the invention is characterised in that it comprises an additional immunoactive component or a nucleic acid encoding said additional immunoactive component

The additional immunoactive component(s) may be an antigen, an immune enhancing substance, and/or a vaccine; either of these may comprise an adjuvant.

The additional immunoactive component(s) when in the form of an antigen may consist of any antigenic component of human or veterinary importance. It may for instance comprise a biological or synthetic molecule such as a protein, a carbohydrate, a lipopolysacharide, a nucleic acid encoding a proteinacious antigen, or a recombinant nucleic acid molecule containing such a nucleic acid operably linked to a transcriptional regulatory sequence. Also a host cell comprising such a nucleic acid, recombinant nucleic acid molecule, or LRC containing such a nucleic acid, may be a way to deliver the nucleic acid or the additional immunoactive component. Alternatively it may comprise a fractionated or killed micro organism such as a parasite, bacterium or virus.

The additional immunoactive component(s) may be in the form of an immune enhancing substance e.g. a chemokine, or an immunostimulatory nucleic acid, e.g. a CpG motif. Alternatively, the vaccine according to the invention, may itself be added to a vaccine.

For instance a vaccine according to the invention can be combined with a preparation of a Babesia subunit vaccine protein, not being a protein of the 28 kDa protein family, to form a combination subunit vaccine against Babesiidae infection or associated clinical signs of disease .

In a yet even more preferred embodiment, the vaccine according to the invention is characterised in that said additional immunoactive component or nucleic acid encoding said additional immunoactive component is obtained from an organism selected from the group consisting of *Ehrlichia canis, Babesia gibsoni, B. vogeli, B. rossi, Leishmania donovani*-complex, Canine parvovirus, Canine distempervirus, *Leptospira interrogans*

serovar canicola, Icterohaemorrhagiae, pomona, grippotyphosa, bratislava, Canine hepatitisvirus, Canine parainfluenzavirus, rabies virus, *Hepatozoon canis* and *Borrelia burgdorferi.*

The protein of the 28 kDa protein family according to the invention, or the immunogenic fragment of said protein, the nucleic acid, cDNA, recombinant molecule, live recombinant carrier, and/or the host cells according to the invention for the first time allow the generation of specific antibodies against a protein of the 28 kDa protein family, or an immunogenic fragment thereof. This makes the vaccine according to the invention suitable as marker vaccine, as it allows the differentiation between parasite infected and vaccinated mammalian targets, through methods known in the art.

Alternatively, these specific antibodies may be used as a vaccine themselves, for so called "passive vaccination".

Therefore still another preferred embodiment relates to a vaccine, characterised in that it comprises an antibody against a protein according to the invention, or an antibody against an immunogenic fragment of said protein, or a combination thereof, and a pharmaceutically acceptable carrier.

A combination in a vaccine of an antigen 'loaded' with antibodies against that antigen is known in the art as a "complex" vaccine.

Still another aspect of the invention relates to a method for the preparation of a vaccine according to the invention, said method comprising the admixing of a protein according to the invention, or an immunogenic fragment of said protein, a nucleic acid, a cDNA fragment, a recombinant DNA molecule, a live recombinant carrier, or a host cell according to the invention, or a combination thereof, and a pharmaceutically acceptable carrier.

Yet another aspect of the invention relates to a method for the preparation of a vaccine according to the invention comprising the admixing of antibodies against a protein or an immunogenic fragment thereof according to the invention, and a pharmaceutically acceptable carrier.

As outlined above, a vaccine obtainable by the method according to the invention can equally be used as prophylactic and as therapeutic treatment, and will interfere both

with the establishment and/or with the progression of an infection or its clinical signs of disease.

Therefore, a further aspect of the invention relates to the use of a protein according the invention or an immunogenic fragment of said protein, for the manufacture of a vaccine for prophylactic or therapeutic treatment of an infection or its clinical signs caused by an organism of the family Babesiidae.

Again a further aspect of the invention relates to a diagnostic test for the detection of a nucleic acid associated with an organism of the family Babesiidae, characterised in that the test comprises a nucleic acid, said nucleic acid being at least 70 % homologous to the nucleic acid sequence depicted in SEQ ID NO: 1 or 3, or a nucleic acid that is complementary to said nucleic acid, wherein either of the nucleic acids have a length of at least 12, preferably 15, more preferably 18 nucleotides.

Yet a further aspect of the invention relates to a diagnostic test for the detection of antibodies against an organism of the family Babesiidae, characterised in that said test comprises a protein according to the invention or an immunogenic fragment of said protein, or a combination thereof.

For instance a Bc28.1 and/or a Bc28.2 protein or an immunogenic fragment of either is coupled to a solid phase carrier, this is incubated with a sample to be tested, is washed, and presence of bound antibodies is detected.

Still a further aspect of the invention relates to a diagnostic test for the detection of antigenic material from an organism of the family Babesiidae, characterised in that said test comprises an antibody against a protein according to the invention or an immunogenic fragment of said protein, or a combination thereof.

For instance antibodies against a Bc28.1 and/or a Bc28.2 protein or an immunogenic fragment of either are coupled to a solid phase carrier, this is incubated with a sample to be tested, is washed, and presence of bound protein is detected.

The invention will now be further described with reference to the following, non-limiting, examples.

Examples

EXAMPLE I: IDENTIFICATION OF THE Bc28 MULTIGENE FAMILY AND MOLECULAR CHARACTERIZATION OF THE Bc28.1 AND Bc28.2 CODING SEQUENCES

1.1. TECHNIQUES USED

1.1.1. General techniques

1.1.1.1. Culture of *Babesia canis*

Isolates of *Babesia canis* (designated A, B, Castres, Gignac, 34.01 and Robin) were obtained from naturally infected dogs from different departments from France. They were maintained in *in vitro* culture according to Schetters *et al.* (1997, Parasitology, vol. 115, p. 485-493).

The A8 biological clone corresponded to a biological clone of the isolate A from *B. canis* and was obtained following an adaptation of the cloning-dilution procedure described for malaria parasites (Walliker & Beale, 1993, Meth. in Molec. Biol., vol. 21, p. 57-66).

1.1.1.2. DNA sequencing

Nucleotide sequencing was performed using the dideoxy chain termination method from alkali-denaturated double-strand templates according to Sanger *et al.* (1977, Proc. Natl. Acad. Sci. USA, vol. 74, p. 5463-5467) by Genome Express S.A. (Zone Astec, Grenoble, France) on both strands of the selected plasmids using T3 and T7 universal primers and various oligonucleotides derived from sequences of each strand already established.

1.1.1.3. DNA primers

DNA primers used for isolating genomic fragments, for generation of probes and for PCR reactions are disclosed in Table 5, with reference to their respective SEQ ID number. All primers were synthesized by Sigma-Genosys (Cambridge, UK).

Name	Sequence (in 5' → 3' orientation)	SEQ ID NO
pr 3	TGATGAAGCCGGCAAGAAGGT	5
E4	TACATGATACCGAATTCAATGG	6
RT1	TTACATCGTTGAGCTCAGCTACCTTGA	7
Inv5	CCATGGATTCAAGGTAGCTGAG	8
5'UTR	AGTCGATACCTCCGAGAATAG	9
Fspe3	ACTGAGGATGAGAACAGGGATAGT	10
Cons3.1	CATGGATTCAAGGTAGCTGAG	11
Rspe4	GACCACAACCGCGACGGCGGCAAC	12
Rspe3G	GAGCTCATTGAGGAGTACAGG	13
Rspe3C	CATTACGCCCACAAATAGTCA	14
3.1expfor	ATTTTGGTTCGTGGATCCACGTGCACTGAGGAT	15
3.1exprevC	CCACAAATAGTCAAGCTTAACCTCTAA	16
3.1exprev	GAATGAGAATCCAAGCTTCTTACCCTTGGC	17
GeneRacer® 5'	CGACTGGAGCACGAGGACACTGA	18
GeneRacer® 3'	GCTGTCAACGATACGCTACGTAACG	19

Table 5: DNA primers used during the course of the experiments

1.1.1.4. Genomic DNA extraction, Southern blot and chromosomal analysis

Genomic DNA extraction from *B. canis in vitro* cultures or from field samples of infected dog blood was performed on 200 µl of blood using Nucleospin® column according to the manufacturer (Macherey-Nagel). Southern blot experiments were performed using standard procedures described in Sambrook & Russell (supra).

The preparation of agarose plugs containing intact or *NotI*-digested chromosomes of *B. canis* and their separation by pulse-field gel electrophoresis (PFGE) were performed as described in Depoix *et al.* (2002, Parasitology, vol. 125, p. 313-321).

The Bc28 probe that was used for the DNA hybridisation experiments was obtained by performing a PCR with the combination of primers Fspe3 and Rspe4 using the plasmid carrying the Bc28.1 cDNA as DNA template. The Bc28 probe was labelled using the Nick Translation kit according to the manufacturer's instructions (Boehringer Mannheim) and as described in Depoix *et al.* (supra).

1.1.1.5. RNA extraction and Northern blot analysis

Total RNA extraction, mRNA purification and RNA hybridisation were performed as described in Drakulovski *et al.* (2003, Infect. Immun., vol. 71, p. 1056-1067). The digoxigenin (DIG)-11-UTP-labelled Bc28 antisense riboprobe (complementary mRNA sequence obtained from using the primers Fspe3 and Rspe4) was synthesized according the DIG High Prime® DNA labelling kit (Boehringer Mannheim).

1.1.1.6. PCR amplification

Amplifications were performed in a PTC-100® Programmable Thermal Controller (MJ Research, Inc) as described in Depoix *et al.* (supra), using Accu Taq® DNA polymerase (Sigma).

1.1.2. Identification of the Bc28.2 genomic fragment from *B. canis* by PCR with primers derived from the *Bd37* cDNA of *B. divergens*

Two primers, pr 3 and E4, were used in a PCR set-up on genomic DNA from isolate A of *B. canis*. The conditions of annealing were the following: the annealing temperature was increased by 2°C per cycle from 45°C to 55°C and then 25 cycles of amplification were performed at 55°C. These conditions allowed to amplify a 500 bp genomic fragment, that was designated Bc28.2. The PCR fragment was then cloned in the pCRII-TOPO® cloning vector according the manufacturer's instruction (Invitrogen), and sequenced.

1.1.3. Cloning of the complete Bc28.1 cDNA sequence of *B. canis*

The complete sequence from the cDNA Bc28.1 was determined by RT-PCR using primers derived from the sequence of the Bc28.2 genomic fragment for the first retrotranscription step. For the RT-PCR experiment, the GeneRacer® protocol was applied according to the manufacturer's instructions (Invitrogen) on intact mRNA from *B. canis*. The 5' end sequence of the cDNA (clone 5'-Bc28.1, 5'-RACE PCR product) was determined by using the reverse primer RT1 for the first retrotranscription step. The 3'-end of the cDNA Bc28.1 (clone 3'-Bc28.1, 3'-RACE PCR product) was obtained using the forward primer Inv5, derived from the sequence of the clone 5'-Bc28.1, in the first retrotranscription step of the protocol. The 5' and 3' ends of the cDNA Bc28.1 were then amplified by PCR using a primer-couple corresponding to the one that was used first for each of the retrotranscription steps and with the forward GeneRacer® 5' primer (5'-

CGACTGGAGCACGAGGACACTGA-3') or the reverse GeneRacer® 3' primer (5'-GCTGTCAACGATACGCTACGTAACG-3') (Table 5) provided in the GeneRacerTM kit (Invitrogen). In both cases, a single PCR product was obtained using Accu Taq® DNA polymerase (Sigma). These fragments were cloned in pCRII-TOPO, and sequenced. Sequences from the clones 5'-Bc28.1 and 3'-Bc28.1 were assembled to form the complete sequence of the Bc28.1 cDNA from *B. canis*.

1.1.4. Cloning of the encoding regions from the Bc28.1 and Bc28.2 coding sequences from *B. canis*

A specific reverse primer from the Bc28.1 and Bc28.2 nucleotide sequences was designed in order to sequence their corresponding genomic copy. The complete open reading frame (ORF) from the coding sequence Bc28.2 was amplified by PCR with the forward 5'UTR primer derived from the 5' *end* of the Bc28.1 cDNA sequence and the reverse Rspe3G primer that specifically hybridises to the 3' end of the genomic sequence of Bc28.2. The sequence of the complete ORF from the Bc28.1 coding sequence was amplified by PCR with the 5'UTR primer and with the reverse Rspe3C primer that specifically hybridises to the 3' end of the Bc28.1 cDNA sequence. The PCR amplifications were performed using the genomic DNA from the biological clone A8 from *B. canis* as DNA template. Amplifications were performed with the following conditions: a 3 min step of denaturation at 94°C, a 3-step cycling program consisting of 1 min denaturation at 94°C, 1 min annealing at 55°C, and 1 min of extension at 72°C, finally followed by a 5 min step at 72°C. The PCR fragments were then cloned in pCRII-TOPO and sequenced.

1.1.5. Analysis of the polymorphism of the Bc28.1 and potential Bc28.2 coding sequences

The Bc28.1 and Bc28.2 coding sequences from the French laboratory Babesia canis isolates B, Robin, Castres, Gignac and 34.01 were amplified by PCR with the primer-couples Fspe3/Rspe3C or Fspe3/Rspe3G and a restriction map based on 5 restriction enzymes (*AluI, EcoRI, HinfI, MboI* and *MspI*) was calculated for both genes. On the basis of the deduced restriction maps from the Bc28.1 coding sequence, the entire coding region of Bc28.1 from the French laboratory *Babesia canis* isolates B, Robin, Castres and 34.01 were amplified by PCR with the primer-couples 5'UTR/Rspe3C, and cloned and sequenced as described above (§ 1.1.2).

1.2. RESULTS:

1.2.1. Identification of a genomic fragment from *B. canis* by PCR

Two primers, pr 3 and E4 were used in a PCR set-up with an increasing annealing temperature from 45 to 55°C on genomic DNA from *B. canis* (Figure 5).

It allowed the amplification of a fragment of approximately 500 bp (Figure 5, lane 2; indicated with a dot). This amplification was specific since the test of each of the primers separately in control amplifications was negative (Figure 5, lanes 5 and 6).

This clone, Bc28.2, hereafter called Bc28.2 (see below) was cloned in a pGEX® vector to be able to express a GST fusion-protein.

1.2.2. Identification of the Bc28 multigene family and cloning of the Bc28.1 and Bc28.2 coding sequences

1.2.2.1. Cloning of the complete Bc28.1 cDNA

In order to find a complete cDNA sequence corresponding to the genomic fragment of Bc28.2, an RT-PCR experiment on intact mRNA from *B. canis* was performed using the GeneRacer protocol. Firstly, a single 5'-RACE PCR product was obtained by performing the first retrotranscription step with the reverse primer RT1 derived from the sequence of the genomic clone Bc28.2 followed by a PCR with the same RT1 primer and the forward GeneRacer 5' primer provided in the Gene Racer kit. Then, a single 3'-RACE PCR product was obtained using the forward primer Inv5, derived from the deduced sequence of the 5'-RACE PCR product, in the first retrotranscription step of the protocol followed by a PCR with the same primer and the GeneRacer 3' primer provided in the kit. Sequences from the 5'-RACE and 3'-RACE PCR products were assembled and constitute the complete sequence of the Bc28.1 cDNA. This cDNA contained a 1039 bp sequence with a poly(A)20 tail and with an ORF of 753 nucleotides. Within the segment 5'UTR/Rspe3C of the cDNA, this ORF starts with an ATG initiation codon at nucleotide (nt) position 50 (or nt position 71 from the entire cDNA) and ends with a TAA stop codon at nucleotide position 820 (or nt position 841 from the entire cDNA).

Alignment of the Bc28.2 genomic fragment sequence with the complete sequence of the deduced cDNA revealed a significant identity in the region of primers RT1 and Inv5 between the two sequences (*i.e.* in the 5' end of the Bc28.2 sequence). However, their 3'

ends were found to be very distant, suggesting the presence of at least 2 related genomic copies in the genome of *B. canis* for this gene. As the cDNA sequence encodes a polypeptide of around 28 kDa, and because its 3' end was distant from the related genomic clone, it was designated Bc28.1 whereas the related genomic clone was designated Bc28.2.

1.2.2.2. Design of primer for specific PCR amplification of Bc28.1 and Bc28.2 coding sequences

As the comparison of the nucleotide sequences from the cDNA Bc28.1 and the genomic clone Bc28.2 suggested two related genomic copies in the genome of the parasite, specific primers able to amplify each of the copies were designed. The two sequences being the most distant in their 3' end, the primers Rspe3G and Rspe3C, respectively designed to amplify the Bc28.2 and Bc28.1 coding sequence, were therefore selected from this region (Figure 3). To demonstrate their specificity for each coding sequence, they were tested by PCR in combination with the primer Cons3.1 (Figure 3) that is located in the 5' end of the Bc28.2 sequence and that hybridises in a conserved region of the two sequences. Moreover, to certify that the two copies of related coding sequences do not derive from two subpopulations of *B. canis* in the isolate A, the PCR's were performed using the genomic DNA from a biological clone, clone A8 of *B. canis*.

The combinations of primers Cons3.1/Rspe3G and Cons3.1/Rspe3C allowed the amplification of a genomic fragment of some 300 bp for both combinations of primers (Figure 6, A, I and II, lanes PCR). To certify that the primers Rspe3G and Rspe3C hybridise specifically to the Bc28.2 and Bc28.1 coding sequence respectively, the amplified fragments were digested by the restriction enzymes *Hinf*I or *Mst*I (Figure 6, A, I and II, lanes H and M). Comparison of the restriction maps of the two sequences revealed the absence of two *Hinf*I restriction sites (located at position 713 and 777 in the Bc28.1 sequence) and one *Msp*I site (located at position 790 in the Bc28.1 sequence) in the 3' *end* of the Bc28.2 sequence (Figure 6, C; compare the restriction maps from the Bc28.1 coding sequence and the Bc28.2 genomic fragment). As expected, the *Hinf*I and *Msp*I digestion of the amplimer Cons3.1/Rspe3C showed a digestion of the amplified fragment in 3 and 2 fragments (Figure 6, A, I, lanes H and M, respectively). In contrast, the Cons3.1/Rspe3G PCR fragment was not digested by these two enzymes (Figure 6, A, II, lanes H and M), demonstrating that the primers Rspe3G and Rspe3C specifically hybridise to the Bc28.2 and Bc28.1 coding sequences from the biological clone A8 from *B. canis*.

In order to analyse the 5' end of the Bc28.2 coding sequence, a similar PCR-RFLP was performed by using the combinations of primers Fspe3/Rspe3G and Fspe3/Rspe3C (Figure 6, B, I and II). In both cases, the amplimers were digested by the two enzymes and the sizes of the fragments resulting from these digestions show that the 5' end of the Bc28.2 and Bc28.1 coding sequences are conserved (Figure 6, C; compare the restriction maps from the Bc28.1 and Bc28.2 coding sequences). Indeed, the 5' end of the Bc28.2 coding sequence contained the single *Hinf*I site (located at position 308 of the Bc28.1 coding sequence) and two *Msp*I sites (located at positions 367 and 412 of the Bc28.1 coding sequence) from the Bc28.1 coding sequence in a conserved position.

In conclusion: two related Bc28 coding sequences are present in the genome of *B. canis*. Specific primers able to analyse each of the two copies are disclosed. The restriction map comparison shows that both copies contain a conserved 5' end whereas their 3' ends are very distant.

1.2.2.3. Hybridisation experiments with a Bc28.1 probe

The probe used for hybridisation experiments (Southern blot, Northern blot and PFGE analysis) (Figure 7) corresponded to the coding region of Bc28.1 cDNA located between primers Fspe3 and Rspe4 (Figure 3).

1.2.2.3.1. *The Bc28.1 coding sequence belongs to a multigene family.*

First, the results of the Southern blot experiment revealed two hybridised fragments of some 1200 and 1400 bp when the genomic DNA of *B. canis* was digested with the *Rsa*I enzyme (Figure 7, A, II, lane 3, indicated by arrows). As no *Rsa*I restriction site is present in the restriction map of the Bc28.1 cDNA, it demonstrates the presence of at least two related genomic copies, in agreement with the identification of the related sequences of Bc28.1 and Bc28.2.

Then, hybridisation experiments were performed with the Bc28.1 probe on the entire (Figure 7, C, II) or *Not*I-digested (Figure 7, D, II) chromosomes from the *Babesia canis* isolates A (Figure 7, C and D, II-A) and B (Figure 7, C and D, II-B), which had been separated by PFGE. This indicated that at least 10 genomic copies of related Bc28 coding sequences exist in the genome of *B. canis* (Figure 7, D, II) and that these copies are located on the 5 chromosomes from the parasite, in both isolates (Figure 7, C, II). The difference in sensitivity of the hybridisation signals, both for isolates A and B, shows a

sequence polymorphism between the different related Bc28 sequences for a given isolate in that family.

1.2.2.3.2. *Northern blot analysis*

Whereas previously described data show there are at least 10 members related to the Bc28.1 cDNA, hybridisation of the probe on total RNA from *B. canis* revealed a single band of around 1.1 kb mRNA, in agreement with the size of the Bc28.1 cDNA sequence (Figure 7, B, II). Moreover, whereas our data show that the two related coding sequences Bc28.1 and Bc28.2 encode different products (28 and 45 kDa, see example 2, section 2.2.1.), no larger mRNA that encoded the 45 kDa was detected.

In conclusion: the Bc28.1 and Bc28.2 coding sequences belong to a multigene family that is composed of at least 10 members located on the 5 chromosomes of *B. canis*. A single mRNA was detected that encodes a 28 kDa protein, corresponding to the Bc28.1 copy. The larger mRNA encoding the 45 kDa protein corresponding to the band recognized by the α-GST-Bc28.2 antiserum could not be detected; the small amount of protein detected in immunoprecipitation experiments indicates this mRNA is transcribed at a very low level, additionally it shows that the transcription of members from the Bc28 family is regulated.

1.2.2.4. Cloning of the Bc28.2 coding sequence within the biological clone A8 from *B. canis* and comparison of its sequences with the Bc28.1 coding sequence

Specific genomic fragment from the Bc28.2 coding sequence was amplified by PCR using the genomic DNA from the biological clone A8 from *B. canis* as DNA template with the combinations of primers 5'UTR/Rspe3G. The genomic fragment was cloned and sequenced. The genomic sequence Bc28.2 was aligned and compared with the equivalent Bc28.1 coding sequence amplified with the combination of primer 5'UTR/Rspe3C both at the nucleotide (Figure 3) and amino acid levels (Figure 1).

1.2.2.4.1. *Comparison at the nucleotide level*

Such a PCR amplified 845 and 852 nucleotide length sequences for the Bc28.2 and Bc28.1 genomic sequence, respectively (Figure 3). Comparison of the Bc28.1 cDNA and genomic equivalent sequences indicated that no intronic sequence was found in the Bc28.1 coding sequence. The percentage of identity, determined using the BlastN

program as described, between the Bc28.1 and Bc28.2 sequences at the nucleotide level was 94 % when the comparison was performed with all 845 nucleotides from the two sequences (Table 3). However, and as suggested by the previously described comparative restriction map analysis using PCR-RFLP experiment (Figure 6, C), comparison of the two nucleotide sequences revealed a strong conservation at the 5' end whereas their 3' end was polymorphic (Figure 3). Indeed, whereas the percentage of identity at the nucleotide level was of 97 % when the comparison was performed with the first 652 nucleotides from the two sequences, the comparison of the remaining 193 nucleotides from their 3' end revealed only 81 % identity (Table 3).

1.2.2.4.2. *Comparison of restriction maps of the Bc28.1 and Bc28.2 coding sequences*

Specific genomic fragments corresponding to the coding region of the Bc28.1 and Bc28.2 coding sequences between the primers Fspe3 and Rspe3C or Rspe3G were amplified by PCR using the genomic DNA from the biological clone A8 from *B. canis* as DNA template. These genomic fragments corresponding to the two copies from the biological clone A8 were digested by various restriction enzymes in order to compare the restriction maps of the Bc28.1 coding sequence and of the Bc28.2 coding sequence (Figure 8, A) within the A8 biological clone.

As previously showed, such PCR-RFLP analyses revealed that both copies shared some restriction sites, especially in their 5' end. The main differences between the two copies are the lack of two *Hinf*I and a single *Msp*I restriction sites at the 3' end of the Bc28.2 coding sequence. At the 5' end of the two copies, the only difference is the lack of an *Eco*RI restriction site at the 5' end of the Bc28.2 coding sequence (Figure 8, A).

1.2.2.4.3. *Comparison at the amino acid level*

Comparative analysis of the products encoded by the coding sequences of Bc28.1 and Bc28.2 was performed (Figure 1). An ORF of 244 and 256 amino acids was predicted for the Bc28.2 and Bc28.1 genomic sequences, respectively (Figure 1). The two ORF's were different in size since, whereas both begin at the same nucleotide position (position 50), the Bc28.2 ORF finishes with a TGA stop codon at position 784 and the Bc28.1 ORF finish with a TAA stop codon at position 820 of their nucleotide sequences (Figure 3). As previously described, the percentage homology between the two predicted proteins was determined with the complete sequence or with the N-terminal or C-terminal of the

proteins (Table 1), using the BlastP program. Comparison of the two complete proteins (on 244 residues) revealed a global homology of 91%. As previously described, whereas the N-terminal part of the two proteins was found to be very conserved (97 % homology between the first 180 amino acids from the two proteins), their C-terminal parts were found to be more polymorphic, with an homology of 73 % in the last 64 amino acids.

1.2.2.4.4. *Predictive analysis of the Bc28.1 and Bc28.2 encoded products*

The proteins encoded by the Bc28cDNA.1 has a predicted molecular weight of 28.3 kDa (and a pI of 6.24) whereas the protein encoded by the Bc28.2 coding sequence has a predicted molecular weight of 27.5 kDa (and a pI of 9.30).

Analysis of the hydrophobicity profile (Figure 9) revealed no internal hydrophobic sequence for both proteins. However, they both shared an N-terminal hydrophobic segment that corresponds to a signal peptide. A cleavage site is present between the A^{16} and V^{17} residues. In contrast to the Bc28.2 protein, the Bc28.1 protein contained another hydrophobic segment located at the C-terminal end of the protein (Figure 9). This G^{236}-V^{256} hydrophobic segment is a GPI anchor.

In conclusion: The two genomic copies Bc28.1and Bc28.2 from the multigene family are predicted to encode products of around 28 kDa. Both proteins contain a cleavable signal peptide at their N-terminal parts. A GPI anchor is present on the C-terminal part of the Bc28.1 protein, but not at the C-terminal part of the Bc28.2 protein. The two related Bc28.1 and Bc28.2 nucleotide sequences, like their deduced amino acid sequences, are strongly conserved in their 5' moiety, but are more polymorphic in their 3' moiety.

1.2.3. Polymorphism of the Bc28.1 and Bc28.2 coding sequences between geographically and genetically disparate *B. canis* field isolates

1.2.3.1. Comparison of restriction maps of the Bc28.1 and Bc28.2 coding sequences by PCR-RFLP

Specific genomic fragments corresponding to the coding region of the Bc28.1 and Bc28.2 coding sequences between the primers Fspe3 and Rspe3C or Rspe3G were amplified by PCR using the genomic DNA from the French *B. canis* isolates A8, B, Castres, Gignac, 34.01 and Robin as DNA template. These genomic fragments corresponding to the two copies from the isolates A8, B, Robin, Castres, Gignac and 34.01 were digested by

various restriction enzymes in order to compare the restriction maps of the Bc28.1 and Bc28.2 coding sequences (Figure 8, B, C respectively) between isolates.

1.2.3.1.1. *Comparison of the Bc28.1 coding sequence restriction maps*

This PCR-RFLP analysis of the PCR fragments amplified with the combination of primers Fspe3/Rspe3C revealed an important conservation of the restriction maps between the Bc28.1 coding sequences from the various available isolates (Figure 8, B).

1.2.3.1.2. *Comparison of restriction maps of the Bc28.2 coding sequence*

Like for the Bc28.1 coding sequence, the PCR-RFLP analysis of the PCR fragments amplified with the combinations of primers Fspe3/Rspe3G revealed an important conservation of the restriction maps between the potential Bc28.2 coding sequence from the various isolates (Figure 8, C).

1.2.3.2. Sequencing of the Bc28.1 coding sequences from different isolates of *B. canis*

To confirm the conservation of the Bc28.1 coding sequences between geographically and genetically disparate *B. canis* field isolates, the coding region (*i.e.* between primers 5'UTR and Rspe3C) of the Bc28.1 coding sequence from the isolates A8, Robin, Castres, B and 34.01 were amplified by PCR, cloned, sequenced, aligned and compared both at the nucleotide (Figure 4) and amino acid levels (Figure 2).

1.2.3.2.1. *Comparison at the nucleotide level*

PCR with the of primer-couple 5'UTR/Rspe3C amplified a 852 nucleotide length genomic Bc28.1 sequence for the isolates B, Robin and A, and a 849 nucleotide length genomic Bc28.1 sequence for the isolates 34.01 and Castres (Figure 4). The percentage of identity at the nucleotide level between these Bc28.1 sequences, in pairwise alignments using the BlastN program, is comprised between 100 % (A8 and Robin are the closest) and 97 % (Table 4), indicating a strong conservation of the coding sequence between isolates of *B. canis*.

1.2.3.2.2. *Comparison at the amino acid level*

Comparative analysis of the protein encoded by the Bc28.1 coding sequence from these different isolates was performed (Figure 2, Table 2). An ORF of 256 amino acids was obtained for the isolates B, A and Robin whereas this ORF was 255 amino acids for the isolates 34.01 and Castres (Figure 2). As previously described, the homology between the different Bc28.1 proteins, determined in pairwise alignments using the BlastP program, was very strong, between 100 % (A and Robin) and 97 % (Table 2).

In conclusion: analysis of the restriction maps from the Bc28.1 and Bc28.2 coding sequences from *B. canis* show a strong conservation of each sequence between the different isolates. As all these isolates were collected in France, this conservation might be due to the fact that these isolates all came from the same country, even if they originate from different regions. However, evidence suggested that these isolates are genetically disparate *B. canis* field isolates. Indeed, their chromosomal content analysis revealed that all these isolates have a specific chromosomal profile. Moreover, the analysis of the polymorphism of the Bc28.1 coding sequence by a PCR-RFLP experiment was performed on around 60 blood samples collected from infected dogs in all parts of France and in other European countries (Germany and Hungary). Such analysis with field samples did not show the identification of other restriction patterns than the ones described in this report, in agreement with a good conservation of this coding sequence between geographically and genetically disparate *B. canis* field isolates.

This conservation between the Bc28.1 coding sequences and deduced proteins for the different isolates was confirmed by sequencing. It revealed an homology between the different coding sequences (identity) and encoded proteins of over 96%.

EXAMPLE II: BIOCHEMICAL CHARACTERIZATION OF THE Bc28.1 AND Bc28.2 PROTEINS

2.1. TECHNIQUES USED

2.1.1. Expression and purification of GST-Bc28.2 and His-Bc28.1 recombinant proteins in *E. coli.*

2.1.1.1. Production of His-tagged Bc28.1 recombinant proteins

Two recombinant Histidine tagged-Bc28.1 proteins, without the N-terminal part of Bc28.1, with and without GPI anchor at the C-terminal part, were designated His-Bc28.1C (V^{16}-V^{256}) and His-Bc28.1 (V^{18}-K^{233}) respectively. These proteins were purified by affinity chromatography on Ni-NTA beads under denaturing conditions for the His-Bc28.1C protein or under native conditions for the His-Bc28.1 protein, according to the manufacturer's instructions (Qiagen).

2.1.1.1.1. *His-Bc28.1C*

The Bc28.1C cDNA sequence without its N-terminal part (nt 104-865 from the entire cDNA or nt 83-844 from the 5'UTR/Rspe3c segment) was amplified by PCR using a cDNA library from *Babesia canis* (isolate A) as DNA template. This cDNA library was constructed, as described in Carret *et al.* (1999, Eur. J. Biochem., vol., 265, p. 1015-1021), with the ZAP Express® cDNA Gigapack II® Gold Cloning kit (Stratagene). PCR was performed using internally modified primers 3.1expfor and 3.1exprevC. These primers contain respectively a *BamH*I and *Hind*III restriction site to allow the cloning of the amplified sequence in *BamH*I/*Hind*III digested pQE-30 vector (Qiagen) in frame with the His-tag present in that plasmid. Then, the PCR product was purified by agarose gel electrophoresis, by loading onto a 0.8% agarose gel (electrophoresis grade, Eurobio, France) running in 0.5x TAE (made from 25x TAE stock solution, Euromedex) at 100V. The band corresponding to the desired product was excised from the gel and the DNA was isolated from the gel slices using a gel-extraction Spin kit® (Q-Bio-Gene). It was then digested with *BamH*I and *Hind*III and gel purified again. The resulting fragment was ligated into dephosphorylated *BamH*I/*Hind*III digested pQE-30 vector, by ligation with T4 DNA ligase (MBI Fermentas, France) in 1x ligase buffer (MBI Fermentas) supplemented

with 2mM ATP (Sigma), at room temperature during 3 hours. The ratio vector:insert was usually 1:3, wherein the amount of digested vector used was between 0.5 and 1µg.

The ligation mix was transformed into JM109 supercompetent® *E. coli* cells (Promega). These cells were plated on ampicillin containing agar plates, and colonies were checked for expression of Bc28.1C protein by protein mini-expression and the recombinant protein (His-Bc28.1C; nt 118-838 from the entire cDNA, or nt 98-817 from the 5'UTR/Rspe3C segment; V^{16}-V^{256}) was purified by affinity chromatography on Ni-NTA beads under denaturing conditions. Briefly, a small scale (5 ml) bacterial culture in LB medium was initiated by 10-fold dilution of an overnight culture. After 2h incubation at 37°C with shaking, recombinant protein expression was induced by addition of 1 mM IPTG (Euromedex). After 3h of induction, cells were harvested by centrifugation (15 min, 4000xg) and lysed in 1 ml of denaturing buffer (8 M urea, 1% v/v Triton X-100, 50 mM Tris, pH 8). Lysates were sonicated for 2 minutes with 2 second pulse-pause cycle on ice, and centrifuged (10 min, 15000xg). Clarified lysates were incubated 20 min on ice with occasional shaking in the presence of 50 µl Ni-NTA agarose resin (Qiagen). Loaded resin was washed thrice with 1 ml of washing buffer (8 M urea, 1% v/v Triton X-100, 50 mM Tris, pH 6.3) and protein eluted with elution buffer (8 M urea, 1% v/v Triton X-100, 50 mM Tris, pH 4.5). The presence of recombinant protein was assessed by SDS-PAGE in 12% polyacrylamide gel, which was stained with Coomassie Brilliant blue (CBB) and by Western blot with anti-His tag monoclonal antibody (Qiagen).

Prior to large scale production of His-Bc28.1C protein, one colony positive for Bc28.1C expression was selected to check the correct in frame fusion of the Bc28.1C core with the 6xHis linker.

In conclusion: a bacterial culture was produced by overnight incubation in 2 ml of LB medium, at 37°C with shaking, and plasmid pQE-His-Bc28.1C was isolated using the JetQuick® miniprep kit (Q-Bio-Gene). The correct in frame fusion of the Bc28.1C core with the 6xHis linker was checked by sequencing. Once checked, the Bc28.1C protein was produced to a larger scale with the same protocol.

2.1.1.1.2. *His-Bc28.1*

The Bc28.1 cDNA sequence deleted from both the N and C-terminal parts (nt 104-787 from the entire cDNA or nt 83-766 from the 5'UTR/Rspe3C segment) was amplified by PCR using internal modified primers 3.1expfor, and 3.1exprev as described above. These primers respectively contain *BamH*I and *Hind*III restriction sites to allow the cloning of the amplified sequence in *BamH*I/*Hind*III digested pQE-30 vector in frame with the His-tag.

The ligation mix was transformed into *E. coli* cells, cells were plated, and colonies were checked for expression of Bc28.1 protein by protein mini-expression, as described above. The recombinant protein (His-Bc28.1; nt 118-769 from the entire cDNA or nt 83-766 from the 5'UTR/Rspe3C segment; V^{16}-K^{233}) was purified by affinity chromatography on Ni-NTA beads under native condition. Briefly, a bacterial culture in LB medium was initiated by 10-fold dilution of an overnight culture, after 2h incubation at 37°C with shaking, recombinant protein expression was induced by addition of 1 mM IPTG (Euromedex). After 3h of induction, cells were harvested by centrifugation (15 min, 4000xg) and resuspended in Histag lysis buffer containing 1% Triton X-100, 1 mg/ml lysosyme and 1 mM phenyl-methyl-sulphonyl fluoride (PMSF) (Sigma). Lysate was stored at -80°C until use. After thawing, 500 U DNAse I enzyme (Life Technologies) was added, incubated 20 min on ice, next the suspension was sonicated on ice for 2 min with 2 second pulse-pause cycles. The sonicate was centrifuged (20 min, 9000xg) and the supernatant was filtered sequentially through 1.2, 0.45 and finally 0.22 µm filters (Pall Gelman, France). Finally, the filtrate was separated on FPLC Ni^{2+} HiTrap® columns (Pharmacia). The loaded column was washed with Histag lysis buffer supplemented with 20 mM imidazole (Sigma). The recombinant Bc28.1 protein was finally eluted in Histag lysis buffer containing 200 mM imidazole.

The His-Bc28.1C protein was purified under denaturing conditions and was injected into a rabbit to produce a polyclonal serum (α-His-Bc28.1C) (section 2.1.2.), which antiserum was used for invasion reduction assays (Example 4).

The His-Bc28.1 protein was purified under native conditions and was used for the erythrocyte binding assay (section 2.1.4.).

2.1.1.2. Production of a GST-tagged Bc28.2 recombinant protein

A GST-Bc28.2 recombinant protein was produced by subcloning the 3' end part of the Bc28.2 coding sequence in frame with GST. Firstly, the recombinant PCRII-TOPO cloning vector carrying the partial Bc28.2 sequence was digested with the restriction enzymes *Bam*HI/*Eco*RI (the 5'*Bam*HI site is from the vector and the 3'*Eco*RI site is from the primer E4). This *Bam*HI/*Eco*RI fragment was purified by agarose gel electrophoresis as previously described, it was excised from the gel and the DNA was isolated from the gel slices using a gel-extraction Spin kit® (Q-Bio-Gene). The resulting fragment was ligated into the dephosphorylated *Bam*HI/*Eco*RI digested pGEX-4T3 vector (Amersham-

Pharmacia), by ligation with T4 DNA ligase (MBI Fermentas, France) in 1x ligase buffer (MBI Fermentas) supplemented with 2mM ATP (Sigma), at room temperature during 3 hours. The ligation mix was transformed into *E. coli* BL21 cells (Novagen) and these cells were plated on ampicillin containing agar plates overnight. A positive colony was selected using PCR with the primers pr 3/E4 and it was induced by IPTG to check for expression of GST-Bc28.2 protein. Thus, the positive bacterial culture was grown overnight and diluted 1:10 with LB medium supplemented with ampicillin at 50 µg/ml. The culture was incubated for 1h at 37°C and then incubated with 0.1 mM IPTG during 3h. Cells were harvested by centrifugation (15 min, 4000xg) and lysed by sonication in MTPBS (150 mM NaCl, 16 mM Na_2HPO4, 4 mM NaH_2PO4, pH7.3) with 1%Triton X-100. The recombinant protein was purified by affinity chromatography on glutathione-agarose beads (Sigma) and eluted by competition with reduced glutathione, i.e. in a buffer containing 50 mM Tris (pH 8) with 45 mM glutathione (Smith & Johnson, 1988, Gene, vol. 67, p. 31-40).

2.1.2. Immunisation

A polyclonal antiserum raised against the GST-Bc28.2 or against the His-Bc28.1C protein was produced respectively in mice and in rabbits according to E. Harlow & D. Lane ("Antibodies: a laboratory manual", ISBN 2907516159). Rabbits (New Zealand White) were immunized with 50 µg of purified His-Bc28.1C recombinant proteins emulsified in Freund's Complete Adjuvant (FCA) (Sigma) subcutaneously for the first injection, and by intramuscular injection for two subsequent times using Freund's Incomplete Adjuvant (FIA) (Sigma). Balb/C mice were intraperitoneally immunized with 15 µg of GST-Bc28.2 emulsified in FCA for the first injection and in FIA for the two subsequent times. Injection of the animals was performed at 3-week intervals and the bleedings were done 8 days before each immunisation.

The rabbit α-His-Bc28.1C antiserum was used to reduce Babesia parasite invasion into erythrocytes, see Example 4.

2.1.3. Immunological methods

2.1.3.1. Immunoblotting

Immunoblotting was performed with purified *B. canis* merozoites prepared as described in Drakulovski *et al.* (2003, Infect. Immun., vol. 71, p. 1056-1067). The merozoites were then processed for electrophoresis and the proteins were separated by 15% SDS-PAGE.

Immunoblottings were revealed using a 1:100 dilution of the polyclonal serum α-His-Bc28.1C.

2.1.3.2. Indirect immunofluorescence assays (IFA)

IFA were performed on *B. canis* parasitised erythrocytes (5% of parasitaemia) as described in Drakulovski *et al.* (supra) using a 1:100 dilution for the polyclonal serum α-His-Bc28.1C. Slides were mounted with Citifluor® solution (Citifluor Ltd, London, UK) for limiting extinction fluorescence and the fluorescence was detected using a fluorescence microscope (Axioscope, Zeiss).

2.1.3.3. [^{35}S]-methionine or [^{3}H]-ethanolamine radiolabeling of *B. canis* culture and immunoprecipitation

[^{35}S]-methionine radiolabeling of *in vitro* cultures of *B. canis* and immunoprecipitation experiments were performed as described in Drakulovski *et al.* (supra). Briefly, the [^{35}S]-methionine-radiolabeling was performed with 50 µCi/ml (1200 Ci/mmol, Amersham-Pharmacia Biotech) and a 5% starting parasitaemia. Fractions used from *B. canis* for immunoprecipitation experiments were: total, culture supernatant (SPA), infected-erythrocyte stroma, purified merozoite, Triton X-114 aqueous (soluble and hydrophilic antigens), and Triton X-114 detergent (insoluble and hydrophobic antigens) fractions, as source of radiolabeled antigens (10^6 cpm). The [^{3}H]-ethanolamine radiolabeling of the *in vitro* culture from *B. canis* was performed with 50 µCi/ml of radiolabeled component (25 µCi/mmol, Amersham-Pharmacia Biotech) with a 5% starting parasitaemia and immuno-precipitation experiments were performed with the total and SPA fractions as the source of [^{3}H]-ethanolamine radiolabeled antigens (10^6 cpm) from *B. canis*.

2.1.3.4. Protein phase separation by TX-114 treatment

The proteins of *B. canis* from the [^{35}S]-methionine radiolabeled merozoite and infected erythrocyte stroma fractions were phase separated in Triton X-114 (Sigma) as described in Precigout *et al.* (1991, Infect. Immun., vol. 59, p. 2799-2805).

2.1.4. Erythrocyte binding assay

100 µg of His-Bc28.1 protein or His-GST (as a control) were purified under native condition according to the manufacturer's instructions (Qiagen). They were then incubated with 50 µl of canine red blood cells in 1 ml of PBS during 1h at room temperature. Erythrocytes were harvested by centrifugation (2 min, 200xg) and resuspended in 200 µl of PBS. The suspension was then layered onto a 400 µl silicon oil cushion (Aldrich). After centrifugation (4 min, 3000xg), the supernatant and the silicon oil were discarded and the proteins bound to erythrocytes were eluted by 75 µl of 0,5 M of PO_4NaCl. Eluted proteins were resolved by SDS-PAGE and detected by an anti His-tag monoclonal antibody (Qiagen) at a 1/20.000 dilution in Western blot using the SuperSignal West Pico Chemoluminescent Substrate kit according to manufacturer's instructions (Pierce).

2.1.5. Analysis of parasitic antigens localised on the surface of *B. canis*-infected erythrocytes

An *in vitro* culture of *B. canis* was firstly radiolabeled with [^{35}S]-methionine as previously described. Erythrocytes were then collected and were biotinylated in a solution of EZ link® sulfoLC NHS biotin (Pierce) (1M in PBS, pH 7.2) during 30 min at room temperature. Then, erythrocytes were washed three times with PBS and the biotinylated extract was passed on a silicone oil cushion (Aldrich), to eliminate lysed erythrocytes. After a centrifugation (20 min, 700xg), intact erythrocytes from the pellet were washed with PBS, lysed and processed for immunoprecipitation experiments as described in Drakulovski *et al.* (supra). Immunoprecipitations were performed with the α-His-Bc28.1C antiserum as previously described or with vaccinated/challenged serum. These vaccinated/challenged sera had been produced by giving dogs three vaccinations with an SPA, followed by a homologous challenge. Such sera had been produced for *B. canis* isolate A (α-A), isolate B (α-B), and *B. rossi* isolate F (α-F) parasites. The corresponding pre-immune dog sera were also tested as negative controls. Then, immunoprecipitated proteins were separated by SDS-PAGE and the gel, rather than to be treated for revelation of immunoprecipitation experiments, was blotted on a nitrocellulose membrane. Biotinylated proteins from the surface of erythrocytes were then revealed by incubating the membrane with a Streptavidin-POD component (Roche) at a 1/2000 dilution and by using the SuperSignal® West Pico Chemoluminescent Substrate kit according to the manufacturer's instructions (Pierce). Once the total biotinylated proteins from the surface of the red blood cells were

revealed, the Western blot was autoradiographed on Biomax MR film (Eastman Kodak Co) in order to certify the parasitic origin of the biotinylated proteins.

2.1.5.1. Determination of the strength of erythrocyte-membrane binding

In an attempt to analyse if the potential surface antigens from *B. canis* immunoprecipitated by the α-His-Bc28.1C antiserum were only attached stuck on the surface of the erythrocytes rather than being a true surface integrated antigen, the strength of their interaction with the surface of infected erythrocytes was evaluated. Erythrocytes from an *in vitro* culture of *B. canis* were radiolabeled, biotinylated and passed through a silicon oil cushion, as previously described. Intact erythrocytes were collected, incubated with an equal volume of NaCl at a concentration varying from 0.5 to 2M during 2 min at room temperature. Then, the eluate was analysed in Western blot with the α-His-Bc28.1C or with the pre-immune rabbit serum as control.

2.2. RESULTS

2.2.1. Biochemical characterization of the Bc28.2 protein

The 504 bp Bc28.2 clone, was cloned in the pGEX vector to produce a purified GST-Bc28.2 recombinant protein of around 35 kDa (Figure 10, A) that was used to produce a polyclonal antibody in mice. This α-GST-Bc28.2 serum reacts weakly but specifically in immunoprecipitation with a 45 kDa protein in the total fraction (Figure 10, B, lane 3, indicated by an asterisk). This 45 kDa protein was also detected by the serum in the merozoite fraction but not in the stroma and SPA fractions of *B. canis* (data not shown). As controls, an unrelated anti-GSTBcvir15 was reactive only with its 15 kDa protein (Figure 10, B, lane 1) and the pre-immune sera were negative (Figure 10, B, lanes 2 and 5). The 28/26 kDa doublet of proteins immunoprecipitated by the α-His-Bc28.1C were never immunoprecipitated by the α-GST-Bc28.2 serum. Similar results were obtained with isolate B of *B. canis*.

In conclusion: the α-GST-Bc28.2 serum reacts with a 45 kDa protein but not with the 28/26 kDa protein doublet recognized by the α-His-Bc28.1C serum.

2.2.2. Biochemical characterization of the Bc28.1 protein

Two recombinant Histidine tagged-Bc28.1 proteins, without the N-terminal part of Bc28.1, but with or without the GPI anchor at the C-terminal part, designated His-Bc28.1C (V^{16}-V^{256}) and His-Bc28.1 (V^{16}-K^{233}) respectively, were purified (Figure 12). The His-Bc28.1C protein was purified under denaturing conditions and was injected into a rabbit to produce a polyclonal serum (α-His-Bc28.1C). The His-Bc28.1 protein was purified under native conditions and was used for the erythrocyte binding assay (section 2.1.4).

2.2.2.1. The Bc28.1 protein is a GPI anchor protein

A metabolic labelling of a *B. canis* lysate with [^{3}H]-ethanolamine, confirmed the GPI nature of the hydrophobic C-terminal peptide of Bc28.1 product since a single ^{3}H labeled protein of 28 kDa was specifically immunoprecipitated with the α-His-Bc28.1C serum (Figure 13, C, lane 2). The pre-immune serum was unreactive (Figure 13, C, lane 1).

2.2.3. Reactivities of the α-His-Bc28.1C serum in immunoprecipitation assays

2.2.3.1. Reactivity on total antigens and soluble parasitic antigen (SPA).

A doublet of 28/26 kDa was recognised in the total fraction of *B. canis* by the α-His-Bc28.1C serum but only the 26 kDa protein was detected in the supernatant, *i.e.* SPA fraction (Figure12, A, lanes 2). Moreover, this doublet of 28/26 kDa proteins corresponded to proteins that were specifically recognized by the anti-A vaccinated/challenged serum (Figure 13, A, lanes 3). Pre-immune sera from rabbit or dog were unreactive (Figure 13, A, lanes 1 and 4, respectively).

2.2.3.2. Reactivity on fractionated antigens

[^{35}S]-methionine radiolabeled parasitised red blood cells were lysed with streptolysin. The sample was centrifuged and the supernatant (erythrocyte stroma fraction) was collected. The pellet (merozoite mixed with ghost) was processed through a Percoll gradient to collect enriched fractions of purified radiolabeled merozoites (merozoite fraction). One part of the merozoite and infected erythrocyte stroma fractions was used for phase separation of the proteins in TX-114. Immunoprecipitation experiments with the α-His-Bc28.1C serum were performed using erythrocyte stroma and merozoite fractions and their corresponding

TX-114 aqueous- (soluble antigens, indicated Aq.) and detergent- (insoluble antigens, indicated Det.) fractions, as sources of radiolabeled antigens of *B. canis* (Figure 13, B).

This shows that the α-His-Bc28.1C serum (Figure 13, B, lanes 2) detects the 28 kDa antigen in the merozoite fraction and that this protein is present in the detergent phase, suggesting it's an insoluble antigen (Figure 13, B, lane Det.). In contrast, the 26 kDa antigen is detected in the stroma of infected erythrocyte and this protein is present in the aqueous phase, suggesting it's a soluble protein (Figure 13, B, lane Aq).

Similar results were obtained with the isolate B (data not shown).

2.2.4. Localization of the Bc28.1 protein by immunofluorescence assays

The pattern of fluorescence of the α-His-Bc28.1C serum on fixed infected erythrocytes from the isolate A of *B. canis* (Figure 13, D) shows a merozoite surface labelling (picture II, as indicated by an arrow). Moreover, a strong labelling of vesicles that are present in the stroma of the infected erythrocyte was also obtained (Picture I, indicated by an arrow on the tetrad form).

2.2.5. Recognition of Bc28.1 by a *B. rossi* antiserum

A vaccination/challenge serum against *B. rossi*, isolate F, was used in immunoprecipitation of ^{35}S labeled *B. canis* isolate B total antigens, which antigens had first been separated with TX-114 into a hydrophobic (detergent: Det.) and a hydrophilic (aqueous: Aq.) phase. This heterologous antiserum precipitated the 28 kDa form of Bc28.1 in the hydrophobic phase, and both the 26 and the 28 kDa form in the hydrophilic phase (Figure 11, lanes 3 and 7), all visible just below the indicated 30 kDa marker band location. Positive and negative control antisera are included.

2.2.6. The Bc28.1 protein binds to erythrocytes

The ability of the Bc28.1 protein to bind to the surface of infected erythrocytes was determined by performing an erythrocyte binding assay (Figure 14, A). As presented in Figure 14 (A, lane 2), the His-Bc28.1 protein was detected in Western blot by the anti-His monoclonal antibody, indicating that this protein is able to bind to canine erythrocytes. As control, no reactivity was observed when the test was performed with the unrelated His-GST (Figure 14, A, lane 1).

2.2.7. The Bc28.1 protein is an erythrocyte surface antigen

Biotinylated intact erythrocytes, resulting from a [^{35}S]-methionine radiolabeling of an *in vitro* culture of *B. canis* (isolate A), were lysed and processed for immunoprecipitations experiments with vaccinated/challenged serum against the isolate A of *B. canis* (α-A) or with the α-His-Bc28.1C antiserum, and with their corresponding pre-immune sera. Immunoprecipitated proteins were separated by SDS-PAGE, the gel was blotted on a nitrocellulose membrane and biotinylated proteins from the surface of erythrocytes were revealed (Figure 14, B, II). Once the total biotinylated proteins from the surface of the red blood cells were revealed, the Western blot was autoradiographed in order to certify the parasitic origin of the biotinylated proteins (Figure 14, B, I). Whereas the α-His-Bc28.1C antiserum immunoprecipitated a 28/26 kDa doublet of proteins (Figure 14, B, I, lane 2), this experiment showed that only the 28 kDa protein was biotinylated (Figure 14, B, II, lane 2), indicating that the 28kDa protein from the doublet is an integrated surface erythrocyte antigen of *B. canis*. This biotinylated 28 kDa protein was also immunoprecipitated by the α-A serum (Figure 14, B, II, lane 3). Pre-immune sera were negative (Figure 14, B, lanes 1 and 4).

2.2.7.1. Determination of the strength of erythrocyte-membrane binding

In an attempt to analyse if the 28.1 surface antigen from *B. canis* immunoprecipitated by the α-His-Bc28.1C antiserum was only attached to the surface of the erythrocytes rather than being associated with it, the strength of the interaction with the surface of infected erythrocytes was evaluated.

Radiolabeled and biotinylated intact erythrocytes were treated with an NaCl solution at a concentration varying from 0.5 to 2M. This showed the 28 kDa protein was not eluted from the surface of the erythrocyte even at a 2M concentration of NaCl. This proves that it is a true surface integrated antigen of *B. canis*.

In conclusion: the biochemical characterization of the Bc28.1 protein showed that the α-His-Bc28.1C serum recognized a 28/26 kDa doublet of proteins.

Both the 28 and 26 kDa proteins are recognized by immune serum of dogs infected by *B. canis*, suggesting that they are excellent candidates for a recombinant vaccine against infection with Babesiidae.

The data indicate that the 28 kDa is an insoluble protein with a GPI-anchor. The protein is associated with the surface of the merozoite and the infected erythrocyte. The 26 kDa protein is a soluble parasite antigen (SPA) that was identified in the infected

erythrocytic and supernatant fractions. The characterisation of the 26 kDa protein as a secreted protein is in agreement with the presence of a cleavable peptide signal at the N-terminal part of the Bc28.1 protein and with the presence in vesicles within the erythrocytes stroma as detected by IFA.

Firstly, the data indicates that the Bc28.1 protein binds to erythrocytes, indicating an interaction of this protein with a ligand from the surface of the erythrocyte. As the 28 kDa product was demonstrated to be associated with the surface of the merozoite, it indicates that its infection of an erythrocyte involves this 28 kDa protein.

Secondly, the data shows also that the 28 kDa form is a surface located antigen. This analysis might also detect soluble antigen that attaches to the surface of infected erythrocytes. However, the fact that treatment of intact erythrocytes with a 2M solution of NaCl was unable to elute the Bc28.1 protein and that the biotinylated surface antigen detected is a 28 kDa protein (*i.e.* the form that is not a soluble antigen), proves that the 28 kDa protein is firmly associated with the infected erythrocyte's outer membrane. This is indicative of a function of the protein in the binding and coagulation of (infected) erythrocytes. Indeed, agglutination of infected erythrocytes was already described for *B. canis* (Schetters *et al.*, 1997, *Parasitology*, vol. 115, p. 485-493). Thus the infected erythrocyte's surface located 28 kDa protein binds to an (infected) erythrocyte component in order to form aggregates that enable the parasite to infect new erythrocytes without becoming exposed to the organism's immune system.

The two functions of the 28 kDa form of the Bc28.1 protein deduced from these data (*i.e.* invasion and coagulation) indicate that this protein plays a crucial role in the survival of the parasite. Indeed, these two mechanisms are essential for the parasite to evade the host immune system.

EXAMPLE III: VACCINATIONS WITH Bc28.1 AND Bc28.2 PROTEIN SUBUNIT VACCINES

3.1. TECHNIQUES USED

3.1.1. Animals

Male and female dogs, for instance Beagles of 6 months old, will be housed in the proper facilities. Several groups will be formed of appropriate size, based on random assignment. Blood samples will be taken before the start, and at several times during the experiment. The animal's general health will regularly be checked.

3.1.2. Vaccines

Bc28.1 and/or Bc28.2 protein will be produced for instance in a baculovirus expression vector system or in the Roche in vitro expression system. Proteins will be characterised through Western blots, quantitated preferably by an Elisa, and formulated, preferably with Quil A.

3.1.3. Vaccinations

Dogs will receive a single dose of vaccine, at two time points, with an interval of approximately three weeks. Injections will be subcutaneous.

At weekly interval blood samples will be drawn, to prepare serum, for serological analysis, preferably by Elisa.

At approximately two weeks after the second vaccination a challenge infection will be given, using an appropriate dose of live *B. canis* parasites.

Animals will be monitored for clinical signs of infection for a period of 14 days after challenge infection. Special attention will be given to behaviour, spleen size, size of lymph nodes, colour of the mucous membranes of mouth and eyelid, and the capillary refill time. Clinical scores will be expressed as a numeric value as described in Schetters *et al.*, 1994 (Vet. Parasitol., vol. 52, p. 219-233).

During the challenge observation time, daily blood smears will be prepared from citrated blood, these will be stained, and the number of parasite-infected erythrocytes will be counted. Daily haematocrit measurements will also be made.

After 14 days of challenge infection, dogs will receive chemotherapeutic treatment with Carbesia®, to cure the infection.

EXAMPLE IV: REDUCTION OF INVASION BY BABESIA PARASITES INTO ERYTHROCYTES WITH SPECIFIC ANTIBODIES

A rabbit polyclonal antiserum was used to prove the capability of antibodies specific for Bc28.1 protein to significantly reduce the invasion of Babesia parasites into erythrocytes.

4.1. TECHNIQUES USED

Standard Babesia cultures on dog erythrocytes were performed as described (Schetters et al., 1994, supra).

α-His-Bc28.1C antiserum had been produced as decribed above (section 2.1.2). In that same experiment rabbit pre-immune serum was obtained, wich does not react with Bc28.1 protein (see Figure 13). These sera were added to Babesia parasite cultures, either pure, or mixed 1:1, in the following scheme:

Serum sample nr 1: pure α-His-Bc28.1C antiserum

Serum sample nr 2: α-His-Bc28.1C antiserum and pre-immune serum mixed 1 : 1

Serum sample nr 3: pure pre-immune serum.

Suspension cultures contained 1% (v/v) dog red blood cells of which 1 % was infected with *Babesia canis* parasites.

To triplicates of such suspension cultures 1:10 volume of the serum samples was added (160 µl serum(-mix) to 1.44 ml of culture), resulting in a final amount of the specific α-His-Bc28.1C antiserum in the cultures of 10, 5 or 0 % v/v.

The cultures with the sera were incubated overnight, after which blood smears were prepared to determine the level of parasitaemia, by counting the number of erythrocytes that were parasite-infected by microscopy.

4.2. RESULTS

The results of the parasite invasion reduction assays are presented in Table 6, and depicted in the graph of Figure 15. From this it is evident parasitaemia in erythrocyte-cultures containing α-His-Bc28.1C antiserum was significantly reduced in comparison to cultures with only aspecific (pre-immune) rabbit serum. The reduction of the invasion amounted to 25%, as the level of parasite infected erythrocytes was reduced from 28 to 21 %.

Even in the 1:1 diluted sample of α-His-Bc28.1C serum effectively this same level of invasion reduction was reached, see Table 6

Serum sample	Amount of α-His-Bc28.1C	Parasitaemia in triplicate cultures			Mean	St. dev.
		A	B	C		
1	10%	20%	22%	21%	20.8%	0.9%
2	5%	19%	20%	21%	20.0%	0.9%
3	0%	29%	28%	28%	28.4%	0.7%

Table 6: results of parasite-invasion reduction assays.

Legend to the figures

Figure 1: Amino acid alignment of Bc28.1 and Bc28.2 proteins

Identities between the two sequences are indicated by asterisks and homologies by single or double dots.

For Bc28.2, the signal peptide located at the N-terminal part, and for Bc28.1 the signal peptide and the GPI anchor located at the C-terminal part are bolded. Their cleavable sites are indicated by vertical arrows.

Figure 2 : Amino acid alignment of Bc28.1 proteins from geographically and genetically disparate *B. canis* field isolates.

Identities between the different sequences are indicated by asterisks and homologies by single or double dots.

The signal peptide located at the N-terminal part, and the GPI anchor located at the C-terminal part of the Bc28.1 proteins are bolded.

Figure 3: Nucleotide sequence alignment of the nucleic acids encoding the Bc28.1 and Bc28.2 proteins according to the invention.

Identities between the two sequences are indicated by asterisks.

The positions of the initiation- and stop codons for the Bc28.1 and the Bc28.2 sequences are indicated; the corresponding nucleotides are bolded.

The location and 5'-3' orientation of primers derived from the Bc28.1 and Bc28.2 sequences are indicated by arrows and bolded; for pr 3 there is only a degenerated match.

Figure 4 : Nucleotide sequence alignment of the nucleic acids encoding the Bc28.1 protein from geographically and genetically disparate *B. canis* field isolates.

Identities between the two sequences are indicated by asterisks.

The position of the initiation and stop codon for each of the Bc28.1 sequences are indicated and bolded.

The location and 5'-3' orientation of primers are indicated by arrows.

Figure 5: Identification of the partial Bc28.2 genomic DNA fragment from *B. canis*.

The Bc28.2 genomic DNA fragment was isolated by PCR with primer-couple pr 3 and E4 on genomic DNA from isolate A, of B. canis (lane 1). As negative controls, each of the primers pr 3 and E4 was also tested (lanes 2 and 3, respectively).

Figure 6: Analysis of the specificity of reverse primers Rspe3C and Rspe3G for their respective Bc28.1 and potential Bc28.2 coding sequences .

PCR's were performed by using the A8 biological clone of B. canis as DNA template. The specificity of the reverse primer Rspe3C for the Bc28.1 coding sequence (I) and of the reverse primer Rspe3G for the potential Bc28.2 coding sequence (II) was tested by their use in a PCR reaction with the forward primer Cons3.1 (A) or with the forward primer Fspe3 (B). Amplimers resulting from these PCR reactions (lane PCR) were then digested with the restriction enzyme HinfI (H) or MstI (M). (C) Recapitulative scheme of the deduced restriction maps of the Bc28.1 coding sequence, partial genomic fragment Bc28.2 and Bc28.2 coding sequence.

Figure 7: Molecular identification of the Bc28 multigene family by hybridisation experiment.

The PCR fragment FSpe3/Rspe4 from the Bc28.1 cDNA sequence was used as a probe for hybridisation experiments. (A) Southern blot of genomic DNA from the isolate A of B. canis digested with the restriction enzymes XbaI (lanes 1), XhoI (lanes 2), RsaI (lanes 3), NotI (lanes 4) and EcoRI (lanes 5). (B) Northern blot of total RNA from the isolate A of B. canis. (C and D) PFGE separation of entire (C) or NotI-digested (D) chromosomes of the isolates A and B of B. canis. (I) Ethidium bromide staining of the gel. (II) Corresponding gels hybridised with the Bc28.1 probe.

Figure 8: Recapitulative scheme of comparative restriction maps of the Bc28.1 and Bc28.2 coding sequences.

(A) Comparative restriction maps of the Bc28.1 and Bc28.2 coding sequences between themselves within the biological clone A8 from *B. canis*. (B) Comparative restriction maps of the Bc28.1 coding sequence between geographically and genetically disparate *B. canis* field isolates. (C) Comparative restriction maps of the potential Bc28.2 coding sequence between geographically and genetically disparate *B. canis* field isolates. The restriction maps were performed on the basis of DNA digestion with *AluI*, *EcoRI*, *HinfI*, *MboI* and *MspI*.

Figure 9: Predictive hydrophobicity profile of the Bc28.1 protein

The predicted N-terminal signal peptide and C-terminal GPI anchor are boxed.

Figure 10: Identification of the protein encoded by the Bc28.2 coding sequence of *B. canis*

(A) SDS-PAGE of the purified GST-Bc28.2 recombinant protein. (B) Reactivity of the α-GST-Bc28.2 on protein extract from the total fraction of [^{35}S]-methionine radiolabelling of an *in vitro* culture of *B. canis* (isolate A). Immunoprecipitations were performed with the immune α-GST-Bcvir15 (lane 1), pre-immune α-GST-Bc28.2 (lane 2), immune α-GST-Bc28.2 (lane 3), immune α-His-Bc28.1C (lane 4), pre-immune α-His-Bc28.1C (lane 5) and immune α-His-Bd37 (lane 6) sera.

Figure 11: Immunoprecipitations of hydrophilic and hydrophobic proteins of *B. canis*.

Total, labeled (Aq.) and detergent-separated (Det.) antigens of *B. canis* isolate B were immunoprecipitated with: a vaccination/challenge serum directed against *B. canis* isolate A (α-A, lanes 1 and 5), an immune serum directed against *B. canis* isolate B (α-B, lanes 2 and 6), a vaccination/challenge serum directed against *B. rossi* isolate F (α-F, lanes 3 and 7), or an uninfected dog serum (N, lanes 4 and 8).

Figure 12: Purification of recombinant His-Bc28.1 proteins.

SDS-PAGE of the purified recombinant His-Bc28.1C and His-Bc28.1 proteins. The His-Bc28.1C protein was purified under denaturing conditions whereas the His-Bc28.1 protein was purified under native conditions.

Figure 13 : Biochemical characterization of the Bc28.1 protein.

Immunoprecipitation experiments of [^{35}S]-methionine (A and B) or [^{3}H]-ethanolamine (C) radiolabeled antigens from *B. canis* (isolate A). Immunoprecipitations were performed with pre-immune α-His-Bc28.1C serum (lanes 1), immune α-His-Bc28.1C serum (lanes 2), immune α-A (from a dog vaccinated/challenged with the isolate A of *B. canis*) serum (lanes 3) and pre-immune α-A serum (lanes 4). Triton-X114 insoluble (Det.) and soluble (Aq.) antigens from the merozoite and stroma fractions were immunoprecipitated with the immune α-His-Bc28.1C serum. (D) Location of the Bc28.1 protein by immunofluorescence assays. Arrows respectively indicate the labelling of vesicles within the stroma of infected erythrocytes (picture I) and of the surface of the merozoite (picture II).

Figure 14: Analysis of the erythrocyte binding property of the Bc28.1 protein and of the surface erythrocyte located antigens of *B. canis*.

Erythrocyte binding assays were performed both with 100 µg of purified His-Bc28.1 protein (lane 2) or His-GST (lane 1) that were incubated with canine red blood cells. The ability of each protein to bind to erythrocyte components was revealed by an anti His-tag monoclonal antibody (Qiagen) at a 1/20.000 dilution in Western blot. (B) Parasitic antigens localised on the surface of *B. canis*-infected erythrocytes were analysed by immuno-precipitation experiments with the α-His-Bc28.1C serum (lanes 1), immune α-His-Bc28.1C serum (lanes 2), immune α-A (from a dog vaccinated/challenged with the isolate A of *B. canis*) serum (lanes 3) and pre-immune α-A serum (lanes 4). Briefly, biotinylated and [^{35}S]-methionine radiolabeled intact erythrocytes were lysed and processed for immunoprecipitation experiments with the vaccinated/challenged serum against the isolate A of *B. canis* (α-A) or with the α-His-Bc28.1C antiserum, and with their corresponding pre-immune sera. Immunoprecipitated proteins were separated by SDS-PAGE, the gel was blotted on a nitrocellulose membrane and biotinylated proteins from the surface of erythrocytes were revealed (II). Once the total biotinylated proteins from the surface of the red blood cells were revealed, the Western blot was autoradiographed in order to certify the parasitic origin of the biotinylated proteins (I).

Figure 15: Results of parasite-invasion reduction assays

Cultures of *Babesia canis* parasites on erythrocytes were incubated or not with different amounts of a polyclonal rabbit antiserum specific for protein His-28.1C. The resulting effect on the parasitaemia was determined by counting the relative number of parasite-infected erythrocytes by microscopy.